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INFLAMMATORY ACTIVATION OF THE KYNURENINE PATHWAY - STUDIES WITH LIPOPOLYSACCHARIDES

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Inflammatory activation of the kynurenine pathway - studies with lipopolysaccharides
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my father and mother

ABSTRACT

Neuroinflammation is increasingly recognised as playing an important role in several major psychiatric disorders. The mechanisms by which neuroinflammation influences neurotransmitter systems have for long been unknown. However, the kynurenine pathway, a source of several neuroactive metabolites, is thought to serve as a link between immune signalling and neuronal activity in the brain. Kynurenic acid (KYNA), an end-metabolite of the kynurenine pathway, is an endogenous N-methyl-D-aspartate-receptor antagonist that strongly regulates brain dopamine activity. Elevated brain levels of KYNA are suggested to be involved in the pathophysiology of psychotic disorders and cognition. The overall aim of this thesis is to investigate the interplay between inflammation and the kynurenine pathway. For this purpose, LPS is administered in doses that induce an inflammatory response in both animals and humans. Our findings show that dual, rather than a single, administration of LPS produces a robust induction of the kynurenine pathway, including increases in brain KYNA levels as well as increased turnover of brain serotonin and dopamine in rodents. Alterations in tryptophan metabolism via the kynurenine pathway in response to dual LPS administration is further shown to induce behavioural impairments, such as cognitive deficits and enhanced amphetamine-induced locomotor activity. In human primary dermal fibroblasts, IFN-γ and IL-1β or their combination, were used to trigger the kynurenine pathway. Forty-eight hours post-stimulation, IL-1β did not elevate extracellular kynurenine and KYNA levels, however IFN-γ induced an 11.5-fold increase in kynurenine and an 8-fold increase in KYNA and the combination of IL-1β with IFN-γ resulted in a synergistic increase in both kynurenine and KYNA. Kynurenine aminotransferase (KAT II) is the main enzyme involved in the synthesis of KYNA. Nevertheless, pharmacological inhibition of KAT II only to some extent reduced the cytokine-induced release of KYNA. In rodents, dual LPS administration increased brain KYNA levels despite pharmacological inhibition of KAT II or genetic ablation of the enzyme. To translate the experimental results obtained in the present thesis to humans, we investigated the effects of systemic LPS administration in healthy human subjects on the kynurenine pathway. Administration of LPS activated both the neurotoxic and the neuroprotective branch of the kynurenine pathway for at least 48 h post LPS-injection.

Overall, the results of the present thesis suggest that the dual LPS model can be used as an animal model, showing both face and construct validity regarding increased central and decreased peripheral KYNA levels as well as regarding aspects of behaviour reflecting psychosis and cognitive deficits. In combination with a cellular assay, this model would be suitable for translational studies of novel immunomodulatory agents, aiming at diminishing KYNA synthesis in psychotic disorders. This thesis, by the means of diverse experimental approaches ranging from the biochemical level, animal behaviour experiments and cellular systems to an experimental human endotoxemia model, confirms the hypothesis of the immune regulation of the kynurenine pathway in psychiatric disorders.

LIST OF SCIENTIFIC PAPERS

I. Markus Larsson*, **Anthi Faka***, Maria Bhat, Sophie Imbeault, Michel Goiny, Funda Orhan, Alfredo Oliveros, Sara Ståhl, Xi-Cong Liu, Doo-Sup Choi, Kristian Sandberg, Göran Engberg, Lilly Schwieler, Sophie Erhardt. *Repeated LPS Injection Induces Distinct Changes in the Kynurenine Pathway in Mice.* Neurochemical Research, 41:2243–2255, 2016

II. Maximilian Tufvesson-Alm, Sophie Imbeault, Xi-Cong Liu, **Anthi Faka**, Doo-Sup Choi, Lilly Schwieler, Goran Engberg, Sophie Erhardt *Repeated Administration of LPS Exaggerates Amphetamine-Induced Locomotor Response and Causes Learning Deficits in Mice.* Journal of Neuroimmunology, 349:577401, 2020

III. Xi-Cong Liu*, **Anthi Faka***, Lilly Schwieler, Robert Schwarcz, Sophie Erhardt. *Systemic administration of LPS increases brain kynurenic acid in mice deficient of kynurenine aminotransferase II* . Manuscript

IV. **Anthi Faka**, Xueqi Li, Sophie Imbeault, Neda Khanlarkhani, Carl Sellgren, Simon Cervenka, Nikolaos Venizelos, Sophie Erhardt, Lilly Schwieler. *Altered levels of kynurenine metabolites following cytokine challenge in primary human fibroblasts*. Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

3-HANA	3-hydroxyanthranilic acid
3-HK	3-hydroxykynurenine
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
α7nACh	α7 nicotinic acetylcholine
AA	Anthranilic acid
AADAT	Aminoadipate aminotransferase
ACMSD	2-amino-3-carboxymuconic-6-semialdehyde decarboxylase
AhR	Arylhydrocarbon receptor
AMPA	a-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ASAT	Mitochondrial aspartateaminotransferase
BBB	Blood-brain barrier
CCBL	Cysteine conjugate beta-lyase
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DOPAC	3,4-dihydroxyphenylacetic acid
GOT	Glutamic-oxaloacetic transaminase
GPR35	G protein-coupled receptor 35
GTK	Glutamine transaminase K
HIV-1	Human immunodeficiency virus type 1
HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine phosphoribosyltransferase
HVA	Homovanillic acid
IC_{50}	Half maximal inhibitory concentration
IDO1	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneal
iPSC	Induced pluripotent stem cell

IS	Internal standards
QPRT	Quinolinate phosphoribosyltransferase
QUIN	Quinolinic acid
KaSP	Karolinska Schizophrenia Project
KAT	Kynurenine aminotransferase
KMO	Kynurenine 3-monooxygenase
KO	Knock out
KYNA	Kynurenic acid
LPS	Lipopolysaccharide
MIP	Macrophage inflammatory proteins
NAD^+	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
PCA	Percloric acid
PIC	Picolinic acid
PLP	Pyridoxal phosphate
SNP	Single nucleotide polymorphism
TBE	Tick-borne encephalitis
TBP	TATA sequence binding protein
TDO2	Tryptophan 2,3-dioxygenase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cells
UPLC-MS/MS	Ultra high-performance liquid chromatography-Mass spectrometry
WT	Wild type
XA	Xanthurenic acid

1 INTRODUCTION

1.1 THE KYNURENINE PATHWAY – PHYSIOLOGY

The essential amino acid tryptophan, important for protein synthesis, serves as the substrate for generating numerous substances with crucial physiological roles. While tryptophan is the origin of serotonin and melatonin synthesis, the kynurenine pathway (Figure 1) is the cardinal biochemical pathway of tryptophan degradation, which is conserved throughout evolution (Ball *et al.*, 2014) and across species. In mammals, 90-95 % of tryptophan is degraded via the kynurenine pathway (Leklem, 1971). The metabolites emerging from the sequential steps of the pathway are responsible for a variety of physiological functions, such as neuronal activation and immune stimulation (Adler *et al.*, 1999; Mándi and Vécsei, 2012; Schwarcz *et al.*, 2012; Stone *et al.*, 2013; Schmidt and Schultze, 2014), as well as regulation of energy homeostasis (Joisten *et al.*, 2020).

The first and rate-limiting step of the pathway is the oxidation of tryptophan to N-formylkynurenine by the heme-dependent enzymes indoleamine-pyrrole 2,3-dioxygenase (IDO1) (Hayaishi, 1976), tryptophan 2,3-dioxygenase (TDO2) (Hayaishi et al. 1957; Tanaka and Knox 1959; Geng and Liu 2014) and the more recently described IDO2 (Ball *et al.*, 2009). N-formylkynurenine is rapidly hydrolysed by kynurenine formamidase (Gál and Sherman, 1978) to produce the central component of the pathway, kynurenine. Further degradation of kynurenine can follow three possible routes. In the first, kynurenic acid (KYNA) is synthesized by irreversible transamination of kynurenine by kynurenine aminotransferases (KAT I-IV) (Han *et al.*, 2010). In the second, kynurenine serves as a substrate for the enzyme kynureninase producing anthranilic acid (AA). In the third route, 3-hydroxykynurenine (3-HK) is formed by the enzyme kynurenine 3-monooxygenase (KMO) and subsequently, 3-hydroxyanthranilic acid (3-HANA) by kynureninase. 3-HANA is also formed from AA through non-enzymatic oxidation (Baran and Schwarcz, 1990). Besides the main branch, 3-HK can be metabolized to xanthurenic acid (XA) by KATs (Sathyasaikumar *et al.*, 2017) and 3-HANA to cinnabarinic acid by non-enzymatic oxidation (Christen *et al.*, 1992). Back in the third route, 3-HANA is converted by 3-hydroxyanthranilate oxygenase to 2-amino-3-carboxymuconic semialdehyde, which non-enzymatically produces quinolinic acid (QUIN). In the final step, QUIN is catabolized by quinolinate phosphoribosyltransferase (QPRT) to nicotinamide adenine dinucleotide (NAD^+) (Henderson 1949; Mehler, Yano, and May 1964), an important cofactor in a variety of metabolic processes. 2-amino-3-carboxymuconic semialdehyde can follow an alternative two-step route and is a substrate for the enzyme 2-amino-3-carboxymuconic-6-semialdehyde decarboxylase (ACMSD) that produces the metabolite 2-aminomuconic-6-semialdehyde, which through a non-enzymatic reaction finally gives picolinic acid (PIC) (Pucci *et al.*, 2007).

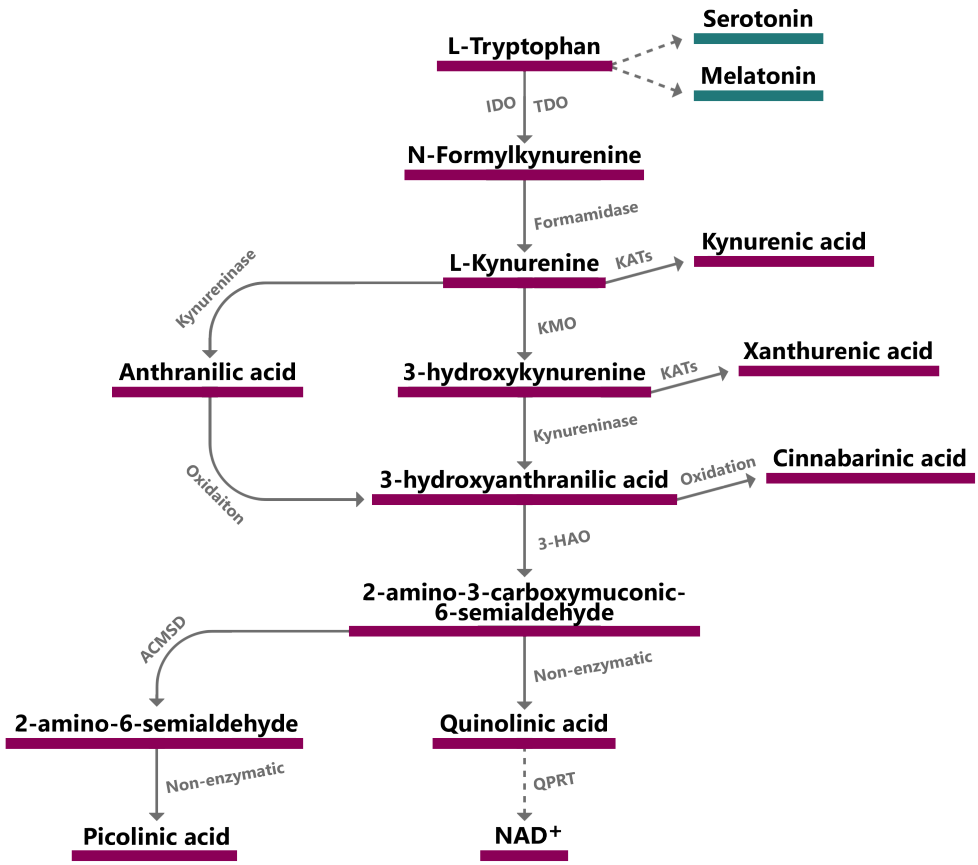

Figure 1. The kynurenine pathway

1.2 THE KYNURENINE PATHWAY IN THE BRAIN AND PERIPHERY

Under normal physiological conditions ~ 90 % of total tryptophan is catabolized by the kynurenine pathway with most of the peripheral tryptophan metabolized by the liver. Of the metabolites that arise from the kynurenine pathway, only tryptophan, kynurenine, and 3-HK can pass the blood-brain barrier (BBB) (Fukui *et al.*, 1991), although there are studies showing that PIC can also enter the brain via olfactory pathways after nasal or systemic administration (Bergström *et al.*, 2002). Changes in the concentrations of peripheral kynurenine pathway metabolites affect the kynurenine pathway in the brain, even though the underlying mechanisms of this apparently bidirectional communication are still unclear (Schwarcz *et al.*, 2012). More specifically, about 60 % of kynurenine is imported into the brain from the periphery (Gál and Sherman, 1978) and taken up by glial cells (Speciale and Schwarcz, 1990) while the rest is derived from local production in the brain. However, these data arise from studies investigating this pathway under physiological conditions. The regulation of the kynurenine pathway can be altered dramatically during immune activation or as a result of different pathophysiological conditions.

All metabolites and enzymes of the kynurenine pathway are present in the brain although their concentration and expression levels can vary between different brain regions, cell types, and species as well as with age (Heyes *et al.*, 1992; Kepplinger *et al.*, 2005; Coggan *et al.*, 2009). Based on astroglial KAT expression, KYNA production is proposed to occur in astrocytes,

whereas microglia express KMO, directing the pathway towards QUIN production (Guillemin, Kerr, Smythe, *et al.*, 2001; Schwarcz *et al.*, 2012) in these cells.

1.2.1 Neuroactive and immune-regulatory properties of kynurenine pathway metabolites

The concentrations of kynurenine pathway metabolites in the central nervous system (CNS) range from nanomolar to low micromolar concentrations (Schwarcz *et al.*, 2012). Importantly for this thesis, several kynurenine pathway metabolites have neuroactive properties. KYNA binds all three ionotropic glutamate receptors, kainate-, a-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA)-, and N-methyl-D-aspartate (NMDA)-receptors (Perkins and Stone, 1982). At lower concentrations, KYNA binds the glycine site of the NMDA receptor (IC_{50}: 8-15 μM) (Parsons *et al.*, 1997), whereas at higher concentrations, the compound acts as an orthosteric antagonist at the glutamate recognition site of the receptor (IC_{50}: 200-500 μM) (Kessler *et al.*, 1989). KYNA also acts as a reversible, competitive antagonist on AMPA and kainate receptors at micromolar concentrations (Kessler *et al.*, 1989). Blockade of α7 nicotinic acetylcholine (α7nACh) receptors (IC_{50}: 7 μM) by KYNA (Hilmas *et al.*, 2001) has been proposed, but this mechanism remains a matter of controversy in the scientific community (Stone, 2020). KYNA also acts as an agonist of G protein-coupled receptor 35 (GPR35) in immune cells and the gastrointestinal tract (Wang *et al.*, 2006), and of the arylhydrocarbon receptor (AhR), which is an intracellular protein acting as transcription factor (DiNatale *et al.*, 2010). Apart from receptor-mediated effects, KYNA serves as an antioxidant by binding to free radicals such as hydroxyl superoxide anion (Lugo-Huitrón *et al.*, 2011). Furthermore, KYNA levels are age-dependent and increase with age in both rodents and humans (Erhardt *et al.*, 2001; Kepplinger *et al.*, 2005). Due to its receptor-binding profile and its ability to reduce glutamate receptor-mediated cytotoxicity, KYNA exerts neuroprotective effects. This can be physiologically relevant during epileptic episodes when elevated KYNA levels can be detected (Majoie *et al.*, 2010). Furthermore, KYNA may limit tissue damage in connection to stroke or other brain injuries (A. Mangas et al. 2017; 2018).

In contrast to KYNA, QUIN acts as an agonist of NMDA receptors (IC_{50}: 180 μM) (Stone, 1993). This, in combination with its ability to promote release and inhibit the reuptake of glutamate in astrocytes (Tavares *et al.*, 2002), provides neurotoxic characteristics to the compound. The production and secretion of QUIN by activated microglia is increased in response to inflammatory stimuli in the brain (Guillemin, Smythe, *et al.*, 2003). Also, QUIN induces astrogliosis and the expression of proinflammatory cytokines and chemokines in human primary astrocytes (Guillemin, Croitoru-Lamoury, *et al.*, 2003; Ting *et al.*, 2009) as well as gliotoxicity (Lee *et al.*, 2010) and astrocytic apoptosis (Guillemin *et al.*, 2005). Another neurotoxic mechanism of QUIN is its involvement in oxidative damage mediated by a complex of QUIN with iron (QUIN-Fe^{2+}) through the formation of reactive oxygen species, which subsequently mediate lipid peroxidation (Goda *et al.*, 1996; Stipek *et al.*, 1997). Additionally, toxicity caused by QUIN can be due to increased production of nitric oxide

through activation of neuronal nitric oxide synthase and inducible nitric oxide synthase in cell models such as human neurons and astrocytes (Braidy *et al.*, 2009). Studies in the rat have shown that the concentration and metabolism of QUIN are age-dependent (Moroni *et al.*, 1984; Stone and Connick, 1985).

PIC has started to attract more attention in recent years and is proposed to balance the neurotoxic effects of QUIN by inhibiting the presynaptic release of glutamate (Vrooman *et al.*, 1993; Beninger *et al.*, 1994). On the other hand, PIC is reported to have antiviral properties by inducing apoptosis of infected cells, which results in reduction of viral replication (Fernandez-Pol *et al.*, 2001). The anti-microbial effects of PIC are mediated by its ability to chelate metal ions such as Fe^{2+} and Zn^{2+} (Cai *et al.*, 2006). The chelating property of PIC is also considered to activate the proinflammatory function of macrophages by inducing the expression of the chemokines macrophage inflammatory proteins (MIP) 1α and 1β (Bosco *et al.*, 2000). Although kynurenine is the pivotal metabolite of the kynurenine pathway, little is known about its neuronal activity. Recent cell culture studies, though, have shown that kynurenine can activate the AhR. This receptor is involved in cell differentiation processes (Yamamoto *et al.*, 2019), tumorigenesis (Opitz *et al.*, 2011) and immune regulation (Mezrich *et al.*, 2010).

Other, less studied metabolites of the kynurenine pathway are cinnabarinic acid and XA both acting as agonists of different glutamate receptors (Fazio *et al.*, 2017), with the latter also acting as an antioxidant by showing high efficiency in scavenging peroxyl radicals, a property shared with 3-HK (Christen *et al.*, 1990). 3-HK is a metabolite raising controversies. Apart from anti-oxidative properties, it can also present toxic effects and more specifically, it is characterised broadly in the literature as an endogenous free radical generator (Colín-González *et al.*, 2013). Recent studies, however, support the association of the antioxidant properties of 3-HK with neuroprotective and anti-inflammatory effects as shown by suppression of neuronal death induced by cytokines in primary human fetal CNS cultures (Krause *et al.*, 2011).

1.2.2 The importance of KATs in the brain

KYNA, the metabolite of interest in our studies, is produced by enzymatic, irreversible transamination of kynurenine, which is catalysed by kynurenine aminotransferases (KATs). This reaction consists of two steps. The KATs are involved in the first step by producing an α-ketoacid that is unstable and rapidly undergoes an intra-molecular cyclization leading to the final product KYNA (Han *et al.*, 2010). All four mammalian KATs show transamination activity towards kynurenine leading to the formation of KYNA. In the CNS of mammals, four isoforms of KATs are identified; KAT I (also known as: glutamine transaminase K (GTK)/cysteine conjugate beta-lyase (CCBL) 1), KAT II (also known as: aminoadipate aminotransferase (AADAT), KAT III (also known as: glutamine transaminase/CCBL 2) and KAT IV (also known as: glutamic-oxaloacetic transaminase (GOT) 2/mitochondrial aspartateaminotransferase (ASAT)) (Okuno *et al.*, 1991; Han *et al.*, 2004, 2010). These isoforms have different physiological and biochemical properties, such as optimal temperature, pH-dependence and substrate specificity and are located in different cellular compartments.

All KAT isoforms are pyridoxal phosphate (PLP) dependent and form homodimers (Jansonius, 1998). Optimum pH conditions (assessed by activity assay) are 7.5-9 for KAT I, around 7 for KAT II, 9-10 for KAT III and 8.5 for KAT VI (Han *et al.*, 2010).

KAT II is the most abundant isoform in the brain and therefore considered the most essential enzyme for *de novo* production of KYNA (Guidetti *et al.*, 2007). Higher concentrations of other competing substrates do not affect KAT II activity and under physiological conditions, KAT II accounts for approximately 75 % of KYNA synthesis in the brain (Chang *et al.*, 2018). Based on the association between abnormal KYNA levels in the CNS, psychotic symptoms and cognitive dysfunctions, the enzymes involved in KYNA synthesis in the brain are considered potential pharmacological targets for regulating brain KYNA levels (Erhardt et al. 2009; Schwarcz and Pellicciari 2002; T W Stone and Darlington 2002; Vamos et al. 2009). Due to its predominant role in the human brain most studies have focused on specific inhibitors for KAT II. So far though, developed KAT II inhibitors have not entered clinical trials due to the toxicity caused by interaction with PLP (Nematollahi *et al.*, 2016). Although the involvement of individual KAT isoforms in KYNA production under normal conditions has been studied, the respective contribution of KAT I-IV in different pathological conditions remains obscure.

1.2.3 Interplay between the kynurenine pathway and the immune system

The kynurenine pathway is critically regulated by the rate-limiting enzymes IDO1 and TDO2. Both are regulated by the immune system where cytokines can affect their expression and activity. TDO2 is not only subjected to corticosteroid and glucagon regulation (Campbell *et al.*, 2014) but can also be induced by interleukin (IL)-1β (Urata *et al.*, 2014; Sellgren *et al.*, 2016). The activity and expression of IDO1 are strongly linked to inflammatory stimuli. IDO1 is expressed in different cells of the immune system, such as monocytes, dendritic cells, and macrophages (Mándi and Vécsei, 2012) and is induced by a number of cytokines such as interferon (IFN)-γ (Hassanain et al. 1993; Munn and Mellor 2016), IFN-α (Wichers and Maes, 2004), IFN-β (Guillemin et al. 2001), tumor necrosis factor-α (TNF-α) (Robinson et al. 2005), TNF-β (Nasef *et al.*, 2007), IL-1-α (Babcock and Carlin, 2000), IL-6 (Hayley, 2011; Litzenburger *et al.*, 2014), IL-2 (Brown *et al.*, 1989), IL-10 (Yanagawa *et al.*, 2009), and IL-27 (Carbotti *et al.*, 2015). These different cytokines can induce IDO1 either alone or synergistically. Contrarily, IL-4 and IL-13 are implicated in the negative regulation of IDO1 (Chaves *et al.*, 2001). Likewise, the KMO enzyme shows an increased expression mediated by IL-1β and IFN-γ (Alberati-Giani *et al.*, 1996; Zunszain *et al.*, 2012), and upregulation of the mRNA for the KAT I/II is correlated with elevated cytokine levels (Kindler *et al.*, 2020). Moreover, studies in human primary dermal fibroblasts show that a cytokine challenge can alter the profile of kynurenine pathway metabolites (Johansson *et al.*, 2013) and induce an increase in transcripts encoding IDO1 after challenge with IFN-γ alone, or in combination with TNF-α (Asp *et al.*, 2011).

While various inflammatory parameters can activate tryptophan metabolism, kynurenine pathway enzymes and metabolites can tune diverse aspects of the immune response. In that frame, IDO1 and other kynurenine pathway metabolites exert an immunosuppressive role. 3-HANA and QUIN induce the apoptosis in T helper 1 cells (Fallarino *et al.*, 2002). Furthermore, kynurenine has immunosuppressive properties via binding to AhR (Opitz *et al.*, 2011) and inhibits Natural Killer cells, induces T-cell death (Mándi and Vécsei, 2012) and also activates regulatory T cells (Treg) (Chen *et al.*, 2008). Moreover, tryptophan depletion is used by host organisms against pathogen proliferation by reduction of intracellular supplies (Moffett and Namboodiri, 2003; Munn and Mellor, 2016). In addition, tryptophan depletion stunts immune reactions via an anti-proliferative and a pro-apoptotic effect on T cells (Lee *et al.*, 2002).

1.3 THE KYNURENINE PATHWAY - PATHOPHYSIOLOGY

Dysregulation of the kynurenine pathway enzymes with subsequent alteration in the metabolite levels is related to a wide range of disorders and symptoms. The metabolism of kynurenines is involved in psychiatric (Erhardt et al. 2017b), neurodegenerative (Maddison and Giorgini, 2015), inflammatory (Badawy, 2018), metabolic (Dadvar *et al.*, 2018) and cardiovascular diseases (Baumgartner et al. 2019) as well as in cancer (Platten *et al.*, 2019) and aging (Sas et al. 2018; Villena 2015).

1.3.1 Implications of the kynurenine pathway in psychiatric disorders

Accumulating evidence points to a dysregulation of the kynurenine pathway in psychiatric disorders. In subjects with schizophrenia, kynurenine and KYNA are elevated in the cerebrospinal fluid (CSF) (Erhardt *et al.*, 2001; Nilsson *et al.*, 2005; Linderholm *et al.*, 2012) as well as in postmortem brain prefrontal cortex (Schwarcz *et al.*, 2001; Sathyasaikumar *et al.*, 2011). Additionally, TDO2 is upregulated in postmortem brains from subjects with schizophrenia (Miller *et al.*, 2004) and bipolar patients with psychotic features (Miller *et al.*, 2006). In a recent meta-analysis, the association between schizophrenia and elevated levels of KYNA in the CNS was described (Plitman *et al.*, 2017). Similar alterations are reported in bipolar patients with a history of psychosis (Olsson *et al.*, 2012a; Lavebratt *et al.*, 2014; Sellgren *et al.*, 2016, 2019). High levels of KYNA in the CSF are also associated with acute psychotic symptoms in HIV-1 infected subjects (Atlas *et al.*, 2007). In a study where primary dermal fibroblasts from patients with schizophrenia and bipolar disorder were cultured, it was shown that more KYNA and 3-HK were released in patient-derived cells compared to those from controls (Johansson *et al.*, 2013). Suicidal behaviour is also linked to aberrations in the kynurenine pathway. Suicide attempters present with increased CSF QUIN levels and increased QUIN/KYNA ratio (Erhardt *et al.*, 2013). Furthermore, reduced levels of the neuroprotective metabolite PIC, as well as the PIC/QUIN ratio in CSF and plasma of suicidal attempters were reported, possibly as a result of a deficiency in ACMSD (Brundin *et al.*, 2016).

The role of the kynurenine pathway in the pathophysiology of depression is receiving increased attention pointing towards an imbalance between KYNA and QUIN (Ogyu *et al.*, 2018). Although previous studies report low levels of KYNA (Myint *et al.*, 2007; Schwieler *et al.*, 2016), it is the relative levels of KYNA to QUIN rather than the absolute concentrations of these metabolites that may be of importance for depression (Savitz *et al.*, 2015). This hypothesis is supported by the finding of increased QUIN/KYNA ratio in plasma (Schwieler *et al.*, 2016) of patients. This ratio may rise with additional episodes of depression (Savitz *et al.*, 2015).

1.3.2 The kynurenine pathway in cognition

Cognitive impairment is tightly connected to alterations in concentrations of kynurenine pathway metabolites (Solvang et al. 2019; Stone and Darlington 2013; Talarowska and Galecki 2015). There are both experimental and clinical studies pointing to the involvement of the kynurenine pathway in cognitive impairment. For instance, subjects with schizophrenia bearing a homozygous risk allele for a single nucleotide polymorphism (SNP) in the KMO gene resulting in reduced activity of KMO performed worse in a range of cognitive tests when compared to control counterparts (Wonodi *et al.*, 2014). In a recent study, it was reported that activation of the kynurenine pathway associates with attention impairments in subjects with schizophrenia. Interestingly, at the same time, proinflammatory cytokines were found elevated in the periphery (Kindler *et al.*, 2020). In most psychiatric and neurodegenerative disorders, cognitive dysfunction is a hallmark of the disease. For example, cognitive deficits are also prevalent in bipolar disorder (Sellgren et al. 2019; 2016) and impaired associative memory is related to plasma kynurenine pathway metabolites in subjects suffering from depression (Chirico *et al.*, 2020).

Indeed, elevated levels of brain KYNA are not only related to psychiatric disorders but also to infectious diseases such as tick-borne encephalitis (TBE) (Holtze *et al.*, 2012), malaria (Holmberg *et al.*, 2017), and human immunodeficiency virus type 1 (HIV-1) infection (Atlas et al. 2007; Baran et al. 2013) as well as to neurological diseases such as Huntington's disease (Jauch *et al.*, 1995) and Alzheimer's disease (Baran et al. 1999; Chatterjee et al. 2018). Also, Down syndrome is associated with increased brain KYNA (Baran *et al.*, 1996). Notably, all of these disorders are also associated with cognitive dysfunctions.

The kynurenine pathway has been studied extensively with regard to cognition in animal models. Pharmacological inhibition of KMO or genetic ablation of this enzyme in rodents leads to an increase in brain KYNA levels resulting in impaired memory and learning (Pocivavsek *et al.*, 2011), disrupted prepulse inhibition (PPI) (Erhardt *et al.*, 2004), and reduced cognitive flexibility (Alexander *et al.*, 2012). In accordance with this, genetic depletion or pharmacological inhibition of KAT II with subsequent decreased levels of KYNA improves memory deficits (Pocivavsek et al. 2019; 2014; Chess et al. 2007; Akagbosu et al. 2012) and cognitive functions in both rodents and primates (Potter et al. 2010; Kozak et al. 2014; Chess et al. 2009; Ortega et al. 2020).

1.4 THE IMMUNE SYSTEM IN PSYCHIATRIC DISORDERS AND COGNITIVE IMPAIRMENT

The demonstration of dysfunction in the immune system in psychiatric diseases gave birth to the scientific field of Neuropsychoimmunology. Epidemiological studies show that early childhood infections (Dalman *et al.*, 2008; Khandaker *et al.*, 2012; Blomström *et al.*, 2014; Karlsson and Dalman, 2020), winter birth (Davies *et al.*, 2003), as well as maternal infection during pregnancy are associated with a larger incidence of schizophrenia (Brown, 2012; Blomström *et al.*, 2016). Clinical studies show elevated CSF levels of IL-1β in patients with first-episode schizophrenia (Söderlund *et al.*, 2009) and elevated CSF levels of IL-6 in subjects with chronic schizophrenia (Sasayama et al. 2013; Schwieler et al. 2015). Postmortem studies report upregulated mRNA levels of IL-1β, IL-6, IL-8, and TNF-α in the brain of patients with schizophrenia (Fillman *et al.*, 2013; Trépanier *et al.*, 2016). In the periphery, concentrations of cytokines IL-6, TNF-α, soluble IL-2 receptor, and the IL-1 receptor antagonist have been shown to be increased in subjects with schizophrenia (Goldsmith et al. 2016). In addition, plasma levels of IL-18 are increased and are associated with cognitive dysfunctions in subjects with first-episode psychosis (Orhan *et al.*, 2018).

With regard to bipolar disorder, the concentration of IL-1β (Söderlund *et al.*, 2011) is increased in the CSF with subjects having a history of psychosis displaying the highest levels (Sellgren et al. 2016). Additionally, mRNA levels of IL-1β, IL-6, and IL-8 are upregulated in postmortem prefrontal cortex in schizophrenia subjects (Fillman *et al.*, 2013). Suicidality is also strongly connected to inflammation (Nässberger and Träskman-Bendz, 1993; Lindqvist *et al.*, 2009; Erhardt *et al.*, 2013; Brundin *et al.*, 2015, 2017). Levels of IL-8 were significantly lower in suicide attempters with anxiety than in healthy controls (Janelidze et al. 2015), while IL-6 levels in CSF were elevated and correlated to the severity of symptoms (Lindqvist *et al.*, 2009).

Although there are few studies investigating CSF cytokine levels in depression, there is a recent study demonstrating that higher levels of IL-1β, IL-23 and IL-33 in CSF are associated with increased odds of perinatal depression (Miller *et al.*, 2019). Furthermore, increased levels of proinflammatory cytokines TNF-α, IL-6, and IL-1β were detected in plasma from patients compared to healthy controls (Dantzer *et al.*, 2008; Dowlati *et al.*, 2010). Notably, more than half of the patients undergoing treatment with IFN-α, for some cancers and viral infections, showed symptoms of major depression (Capuron and Miller, 2004; Raison *et al.*, 2010). In line with this, anti-inflammatory treatment can ameliorate the symptoms of depression (Tyring *et al.*, 2006).

1.5 LPS

Lipopolysaccharide (LPS) is a cell wall component of Gram-negative bacteria. LPS activates different cell types of the immune system by binding to Toll-like receptors (TLRs) (Schletter *et al.*, 1995; Ulevitch and Tobias, 1995), thereby providing the molecular basis for a pattern

recognition response of pathogens. TLRs are type I transmembrane glycoproteins that mediate pattern recognition and elicit immune responses after infection or tissue damage. From the ten different TLR subtypes that are expressed in humans, TLR-4 is involved in signalling induced by LPS.

1.5.1 LPS and immune activation

LPS is widely used in animal and cell experiments to trigger an immune response. Upregulation of the expression of proinflammatory cytokines such as IL-1β, TNF-α, IL-6 (Parrott et al. 2016), and IFN-γ (Dantzer, 2001) follows peripheral LPS challenge in rodents. Moreover, in human monocytes, IL-10 and IL-8 are increased after stimulation with LPS (Finney *et al.*, 2012).

In recent years, LPS-induced endotoxemia was introduced as a model for acute systemic inflammation in humans in order to elucidate the interplay between brain function and peripheral immune responses (Schedlowski *et al.*, 2014). LPS is also used as a tool to study how systemic inflammation affects motivation (Lasselin *et al.*, 2017) and triggers mood disorders (Eisenberger *et al.*, 2010; Cho *et al.*, 2019; Irwin *et al.*, 2019; Kruse *et al.*, 2019), pain perception (Karshikoff *et al.*, 2015, 2016) and sickness behaviour (Henderson et al. 2017; Lasselin et al. 2020; Regenbogen et al. 2017; Sundelin et al. 2015).

In healthy volunteers, a low dose of endotoxin is adequate to increase plasma levels of TNF-α and IL-6 and to induce depression and decrease memory functions (Reichenberg *et al.*, 2001). In rodents, administration of LPS results in a sickness response characterized by reduced locomotor activity, decreased food intake, fever, and reduced social interaction (Dunn *et al.*, 2005). Sickness behaviour lasts 2-6 h after LPS administration while depressive-like behaviours appear within 24–28 h (O'Connor *et al.*, 2009; Salazar *et al.*, 2012; Walker *et al.*, 2013). Experimental studies show that repeated administration of LPS alters blood-brain barrier (BBB) permeability, in particular with regard to cytokines (Banks and Erickson, 2010) by altering transport systems. Consequently, repeated LPS doses induce a more significant inflammatory response than a single injection (Tarr *et al.*, 2012). Thus, brain and serum concentrations of cytokines and chemokines are altered following repeated injections of LPS (Erickson and Banks, 2011). Two injections of LPS, given with an interval of 16 h, induce mRNA expression of IL-12 p40, a cytokine playing a central role in regulating cell-mediated immunity (Stalder *et al.*, 1997).

1.5.2 LPS and the kynurenine pathway

It has been known for decades that LPS upregulates IDO1 mRNA expression, as well as protein expression and enzyme activity, subsequently leading to an activation of the kynurenine pathway (Yoshida and Hayaishi, 1978). Numerous studies have also shown that systemic administration of LPS activates the kynurenine pathway and elicits cognitive deficits (Haba *et al.*, 2012; Ming *et al.*, 2015; Imbeault *et al.*, 2020), anxiety (Camara *et al.*, 2015), and

depressive-like behaviour in rodents (Yirmiya, 1996; O'Connor *et al.*, 2009; Salazar *et al.*, 2012). Studies using mouse primary microglia demonstrate that LPS stimulation increases IDO1 transcription (Connor *et al.*, 2008; Wang *et al.*, 2010; Hemmati *et al.*, 2019). Similar studies indicated that LPS administration resulted in increased L-kynurenine production (Fujigaki *et al.*, 2001, 2006), which is in agreement with findings from our laboratory showing that stimulation of TLR-4 induces kynurenine production in mice (Imbeault *et al.*, 2020) and in human monocyte cultures (Orhan *et al.*, 2016). Depressive-like behaviour is considered to be related to IDO1 activation, subsequently resulting in elevation of QUIN levels (O'Connor *et al.*, 2009; Salazar *et al.*, 2012; Walker *et al.*, 2013; Tao *et al.*, 2020). In line with the above-mentioned experimental data, clinical experimental studies have shown that endotoxins, such as LPS, enhance release of pro-inflammatory cytokines and induce the kynurenine pathway subsequently leading to elevations of kynurenine metabolites in plasma (Padberg *et al.*, 2012; Kruse *et al.*, 2019).

1.5.3 LPS and cognition

Peripheral administration of LPS in animals is linked to cognitive dysfunction (Ming et al. 2015; Czerniawski et al. 2015; Haba et al. 2012; Salazar et al. 2012; Richwine et al. 2009; Sparkman et al. 2006). Furthermore, LPS-induced release of pro-inflammatory cytokines affects neuronal function resulting in the activation of cell signalling pathways that play a role in long-term potentiation (LTP), glutamate release, and AMPA receptor trafficking (Albensi and Mattson 2000; D'Arcangelo et al. 2000; Lynch et al. 2004; O'Connor and Coogan 1999; Vereker et al. 2000; Tancredi et al. 2000). Given that such processes are involved in synaptic plasticity and neurotransmission, one can surmise that cytokines, directly or indirectly, impact cognition. LPS is tightly linked to alterations in metabolic pathways such as the kynurenine pathway (O'Connor *et al.*, 2009; Walker *et al.*, 2013). Notably, IDO1 knockout mice are protected from cognitive and memory deficits induced by LPS (Heisler and O'Connor, 2015) and increased KYNA levels after LPS challenge in mice are related to memory dysfunction and learning deficits. (Oliveros *et al.*, 2017; Peyton *et al.*, 2019; Imbeault *et al.*, 2020)

2 AIMS

Overall aim of the thesis

To investigate the interplay between inflammation and the kynurenine pathway

Specific aims:

1) Investigate the effects of immune stimulation on kynurenine pathway metabolites

- in mice and rats (single and dual LPS treatment)
- in primary human fibroblast cultures (LPS and cytokine stimulation)
- in humans (single LPS treatment)

2) Investigate mouse behaviour following dual LPS treatment

3) Investigate if KAT II inhibition can prevent the synthesis of KYNA following immune stimulation

- in mice and rats
- in primary human fibroblast cultures

3 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

Animal studies:

All animal experiments are based on scientific and ethical grounds and we have placed great emphasis on minimizing the suffering of animals. All behavioural tests are well established and validated. For all animal experiments in this proposal, ethical permits are approved and accepted by the Ethical Committee of Northern Stockholm, Sweden (N55/14, N274/15 and 2546-2019). All animal experiments also follow the Swedish legislations (The Animal Welfare Act, The Animal Welfare Ordinance and Regulations from the Swedish Board of Agriculture) and KI Animal Welfare Body recommendations. All studies are carried out in accordance with the 3Rs: Replacement, Reduction, and Refinement and Directive 2010/63/EU.

Human subject studies:

Human studies are approved by the local ethical review board in Stockholm or Uppsala, Sweden (2010;879-31/1) or the Institutional Review Board of the St. John of God Hospital (Linz) and the ethics committee of the Medical University of Vienna (Nr: 1599/2017). All human experiments are carried out in accordance with the Bioethics Convention (Oviedo) and "The code of ethics of the world medical association" (Declaration of Helsinki). Every individual included in the studies has received verbal and written information about the planned research project and potential complications before providing verbal and written informed consent. All samples are frozen, coded with a unique barcode, and sent to biobanks. We have no information regarding the identity of the included individuals. The code key connecting to personal data is stored separately, encrypted, and will not be used within the framework of our research projects.

3.2 DRUGS AND CHEMICALS

All drugs and chemicals used for the experiments that resulted in the production and collection of the data in this thesis are described below. All drugs were of the highest purity possible.

Animal studies: LPS (Escherichia coli serotype O111:B4, lot # 091M4031V), D-amphetamine and L-kynurenine sulphate salt (all from Sigma-Aldrich), KAT II inhibitor (PF-04859989, Sigma-Aldrich) and isoflurane (Forene®, Abbott Scandinavia).

Isolation, culture and stimulation of primary human fibroblasts: DMEM Glutamax (Cat. # 61965-026), HEPES buffer (Cat. # 15630056), MEM amino acids (Cat. # 11140035), sodium pyruvate (Cat. # 11360039), penicillin-streptomycin (Cat. # 15140-122), fetal bovine serum (FBS, Cat. # 10270106) all purchased from Gibco. Upon stimulation, DMEM medium (Cat. # 21013024) from Gibco, tryptophan (Cat. # 93659) and KAT II inhibitor (PF-04859989, Cat. # PZ0250) both from Sigma-Aldrich and human recombinant IFN-γ (Cat. # rcyec-hifng), and IL-1β (Cat. # rcyec-hil1b), both from InvivoGen, were applied to the cell cultures.

Clinical study: LPS (Escherichia coli O113 (National Reference Bacterial Endotoxin; lot #94332B1, Investigational Drug Management at the National Institutes of Health, Bethesda, Maryland).

Analysis with high-performance liquid chromatography (HPLC): perchloric acid (PCA) sodium acetate and acetonitrile, zinc acetate, methanol, sodium metabisulfite, EDTA, Na_2EDTA, octanesulfonic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) (Sigma-Aldrich).

Analysis with ultra high-performance liquid chromatography-Mass spectrometry (UPLC-MS/MS): Normal standards: tryptophan, L-kynurenine, pyridine-2,3-dicarboxylic acid, QUIN and KYNA (Sigma-Aldrich). Internal standards (IS): tryptophan-d3, L-kynurenine-d4, QUIN-d3, (Toronto Research Chemicals Canada) and KYNA-d5 (C/D/N Isotopes Inc.). Solutions for the mobile phases: methanol and formic acid 99 % all LC-MS grade (Chromasolve, Honeywell, VWR International AB, Stockholm). Solutions for sample preparations: ammonia solution (32 %) (VWR), $ZnSO_4$ (Sigma-Aldrich).

RNA extraction-qPCR: Isol-RNA lysis reagent (5PRIME), amplification grade DNase I and Applied Biosystem Reverse Transcription Kit (Life Technologies), SYBR Green and PCR Master Mix (both Applied Biosystems).

3.3 ANIMAL STUDIES (Paper I-III)

All animals used for our studies were housed at 25 °C under 40–60 % humidity, on a 12-hour lights on/off cycle (lights on at 06:00 am). Food and water were offered *ad libitum* and animal conditions were checked daily. All animals were monitored and weighed pre- and post-LPS injections. Animals showing excess signs of sickness or decreasing more than 15 % in body weight were excluded from the study and sacrificed. At experiment termination, animals were sedated with isoflurane (≈ 4 % in air) and decapitated. Brains were harvested, quickly frozen,

and stored at -80 °C. Blood was collected and stored overnight at 4 °C. The following day, serum was pipetted from the blood samples and centrifuged for 15 min (1500 x g) and the supernatant was transferred to a new Eppendorf tube and stored at -80 °C.

3.3.1 Mice

Animals were housed in groups of 2-7 individuals. Experiments were carried out on 2.5-5-month old adult mice weighing between 30 and 35 g upon entering the studies. A separate cohort of KAT II knock out (KO) mice consisted of animals at postnatal day (P) 22. Male wild type (WT) C57BL6/N, FVB/N and C57BL6/J mice as well as transgenic KAT II KO mice (FVB/N background) used for our studies were bred at our animal facility. KAT II KO mice were generated as previously described (Yu *et al.*, 2004). The reason for using KAT II KO mice at P 22 is that under physiological conditions, KAT II KO mice show reduced KYNA levels in the brain up to P 28. KYNA levels return to normal concentrations in adulthood (Yu *et al.*, 2004).

3.3.2 Rats

Male Sprague Dawley rats were ordered from Charles River laboratory and housed in groups of five individuals at arrival. The rats acclimatized for a minimum of one week prior to the experiments and weighed between 200-300 g when entering the study.

3.4 HUMAN STUDIES (Paper IV, V)

The Karolinska Schizophrenia Project (KaSP) is a multidisciplinary research consortium investigating the pathophysiology of schizophrenia. In the frame of this project, drug-naïve, first-episode psychosis patients and healthy controls are recruited. The recruited subjects undergo a battery of psychiatric and cognitive evaluations. At the end of the testing battery, which is conducted for one week, CSF and blood samples, as well as skin biopsies, are collected. Exclusion criteria are severe somatic or neurological illness, as well as neurodevelopmental disorders or drug abuse. Healthy control subjects are recruited via advertisement and undergo the same sampling procedure. Current or previous psychiatric illness is excluded via an interview and their health status is assessed by their medical history, clinical examination, blood and urine tests as well as brain MRI. Exclusion criteria are first-degree relatives suffering from any psychotic disorder or substance abuse, including alcohol. Eighteen months following recruitment, patients and healthy controls returned for a follow-up evaluation where the same testing and sampling procedures were repeated.

For the needs of the study on paper IV, ten healthy subjects were recruited from the KaSP cohort. From those, seven were males (70 %), 21-42 years old. Another cohort of ten healthy male individuals (not part of KaSP), 18-40 years old, was used in paper V. All subjects in paper V underwent a physical examination, routine laboratory testing, and an

electrocardiogram. All healthy volunteers were non-smokers, free from medication, and without any known history of disease.

3.5 SAMPLE PREPARATION

For the different analyses the samples were prepared as follows:

3.5.1 HPLC (Paper I-III)

In order to analyse the concentration of KYNA and kynurenine in our samples they were prepared as follows: We used one hemisphere of the mouse brain or the upper half of the right hemisphere of the rat brain. Brains were placed in threefold or fivefold (w/v) 0.4 M PCA (0.1 % sodium metabisulfite, 0.05 % EDTA) respectively, and homogenized with a disperser (Ultra-Turrax®, IKA, Stauffen, Germany). The brain homogenates as well as the serum were centrifuged at 21000 x g for 5 min and pure PCA (70 % strength, 1/10 v/v) was added to the supernatant. The samples were then incubated at 4 ^{o}C for at least 30 min and centrifuged again (21000 x g for 5 min). Immediately, the supernatants were transferred to new tubes for analysis. For the analysis of dopamine, 5-HT and their metabolites, 160 µl dH_2O and 20 µl NaOH were added to 20 µl of the supernatant prepared as described for KYNA analysis.

3.5.2 LC-MS/MS (Paper IV, V)

Cell culture supernatant (cleared by centrifugation at 20800 x g for 5 min after thawing and before analysis), brain extract or human EDTA-plasma, calibrator sample or Quality Control sample (30 µl) was mixed with 30 µl of IS (0.5 µM in 10 % ammonium hydroxide solution) for 15 s. 60 µl of $ZnSO_4$ (200 nM) was added and mixed for 15 s and subsequently 30 µl of methanol was added and mixed for 15 s. The mixture was then centrifuged for 10 minutes at 2841 x g at room temperature. 30 µl of the supernatant were mixed with 30 µl of formic acid 5 % (in LC-MS Certified Clear Glass 12 x 32 mm vials) before transferred to an autosampler (5 °C).

3.6 PROTOCOLS

3.6.1 Treatments (Paper I-III)

Animals treated with LPS received two injections intraperitoneally (i.p.) (mice: 0.83 mg/kg + 0.83 mg/kg, rats: 0.5 mg/kg + 0.5 mg/kg) 16 hours apart (Figure 2). Control groups followed the same timeline and received equivalent doses of the vehicle. Animals were sacrificed 24 hours after the first injection (for paper I also after 48, 72 and 120 h). LPS was prepared daily in vehicle (sterile saline) and stored at 4 °C before injection. The KAT II inhibitor (PF-04859989) was administered i.p. in mice (5, 10 or 50 mg/kg) 40-60 min prior to the last LPS or vehicle injection and in rats (10 mg/kg) 40-60 min prior to the first or both LPS injections (Figure 2). Animals were sacrificed 24 h after the first injection. The KAT II inhibitor was prepared daily in vehicle (sterile saline).

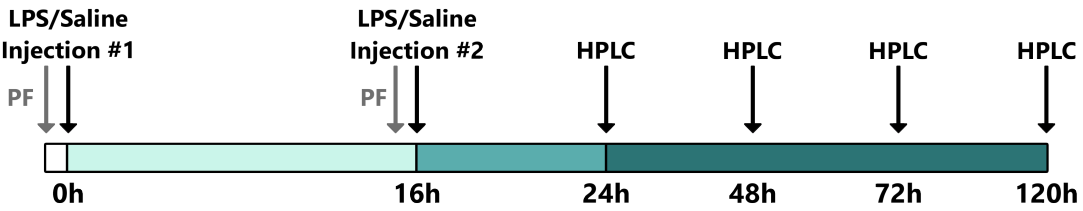

Figure 2. Schematic representation of the treatment protocol (**paper I-III**)

Mice received L-kynurenine (20 mg/kg or 40 mg/kg) or vehicle i.p. Animals were sacrificed 2 h after injection. L-kynurenine was prepared daily in vehicle (sterile saline) and adjusted to pH≈ 8.2.

3.6.2 Behavioural tests (Paper II)

All behavioural animal experiments were performed in mice, 3-4 months old. All tests were performed from 08:30 to 16:00. Every animal was subjected to a minimum of two days of handling by the researcher performing the final test and habituated to transportation to the testing room. Animals were left undisturbed in the testing room for at least 30 min prior to the start of testing. Separate cohorts of animals were used for each test. The design and treatments for the different behavioural test are shown in Figure 3 below.

Locomotor activity test

In order to assess locomotor activity, we used an open-field arena where each mouse was placed in a square Plexiglas box (50 × 50 × 21.6 cm) within a solid, sound-dampened chamber. The chamber was equipped with two rows of photocells (infrared sensitive) forming a two-layer grid over the arena and a computer registered each interruption of a photocell as one count. Horizontal activity, rearing activity and corner time were analysed. Mice were habituated for three days (one session/day, 60 min/session). LPS (0.83 mg/kg) or saline was given after the second habituation and another dose of LPS (0.83 mg/kg) 16h later. During the third session (40 h after the first treatment) general locomotor activity was assessed. Immediately after that, animals were injected with 5 mg/kg D-amphetamine or saline and locomotor activity was recorded again (additional 90 min). No signs of stereotypy were observed. The activity prior to the injection of D-amphetamine (last 5 min, depicted as time 0) was used as a baseline so as to determine the area under the curve (software GraphPad Prism).

Trace fear conditioning

A fear conditioning chamber (Med Associates Inc., St. Albans, VT, USA) was used to perform the trace fear conditioning test. During the training session (40 h after the first LPS or saline injection) mice were allowed to explore the chamber (100 s) until a tone cue (20 s, 90 dB) and

a foot shock (2 s, 0.5 mA) were introduced with a 'trace' interval of 18 s. After a 100 s inter-trial interval, another tone-shock pairing was presented before removing the mouse (30 s after the last shock). After three days, freezing was recorded in the same context, without any tone or shock exposures in order to assess context-dependent memory. Three hours later, freezing was recorded again in response to a cue, by placing the mouse in the same apparatus but in a novel environment (a plastic floor covered the metal grid and a pyramidal shape insert changed the shape of the rectangular box). The cue was introduced (20 s) after exploration (100 s), followed by a second cue (20 s) after an inter-trial interval (120 s). Freezing, defined as the absence of movement (excluding respiration movements), was scored automatically by the software (VideoFreeze v.2.5.5.0; Med Associate) and the percentage of freezing time was used to score learning and memory.

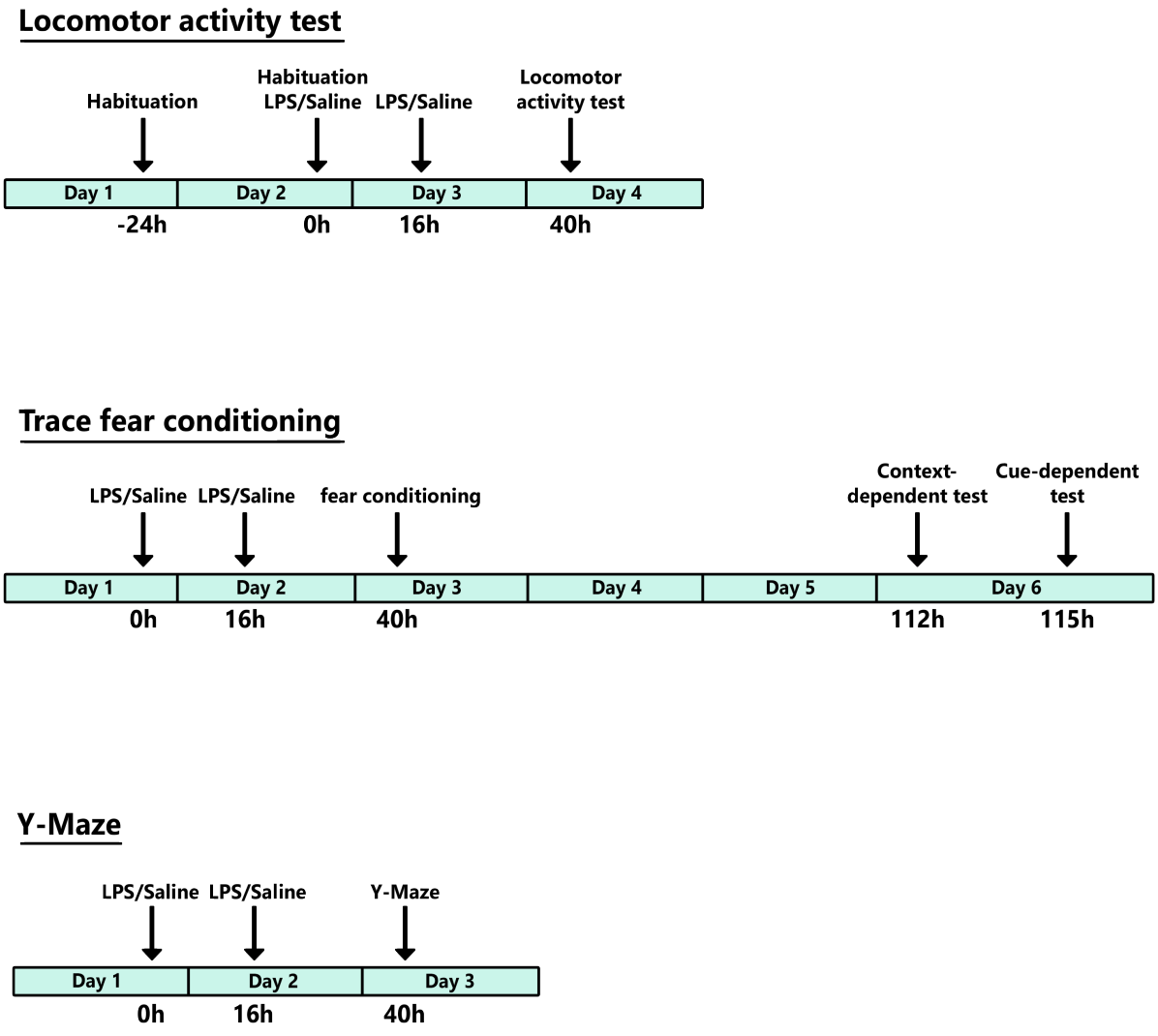

Figure 3. Schematic representation of the design of behavioural tests and LPS treatments in relation to the testing

Y-maze

Working memory was carried out in the Y-maze (PanLab) using the continuous alternation paradigm. Animals were allowed to explore continuously for 5 min after being placed in one arm of the Y-maze. The movement was recorded from above and the calculations were as follows:

Spontaneous alternations: $\frac{number\ of\ alternations \text{ x } 100}{total\ number\ of\ arm\ entries} - 2$

Returns in the same arm: $\frac{number\ of\ returns\ to\ the\ same\ arm \text{ x } 100}{total\ number\ of\ arm\ entries} - 1$

Alternate arm returns: $\frac{number\ of\ alternate\ arm\ returns\ x\ 100}{total\ number\ of\ arm\ entries} - 2$

3.6.3 Tissue isolation and fibroblast culture (Paper IV)

For establishing the human primary fibroblast cultures, a cutaneous biopsy was taken from the upper (inside) arm of participants. Tissue specimens were minced and cultured in 35 mm dishes under a sterile glass coverslip in DMEM Glutamax, supplemented with 10 mM HEPES, 1X MEM amino acids, 1X sodium pyruvate, penicillin (100 U/ml) - streptomycin (100 µg/ml) and 20 % FBS in a humidified 37 °C, 5 % CO_2 incubator. Two passages later, cells were frozen and stored in liquid nitrogen until further use.

3.6.4 Cytokine stimulation (Paper IV)

Following thawing and two passages, cells were seeded into 96-well plates. Once they reached 75 % confluence, the KAT II inhibitor (PF-04859989; 500 nM) or vehicle (saline) was applied in serum-free DMEM medium complemented with 1mM of tryptophan, 10 mM HEPES, 1x sodium pyruvate and penicillin (100 U/ml) - streptomycin (100 µg/ml). Human recombinant IFN-γ (20 ng/ml), IL-1β (10 ng/ml) or their combination, were added to the cells an hour later. Cell culture studies were performed in technical triplicates. Supernatants were collected 48 h after cytokine stimulation and kept at -80 ^{o}C until further analysis.

3.6.5 Human study (Paper V)

The human LPS study is a prospective, randomized, single-blinded, placebo-controlled cross-over study. At 8:00 a.m. and after overnight fasting, intravenous catheters were inserted on each arm of the participants. They received saline (0.9 %) or LPS (2 ng/kg) over 5 min together with saline (0.9 %) (total duration 90 min [200 ml/h]) at two different days (washout period at least 14 days). LPS was reconstituted with sterile water and prepared according to the manufacturer's recommendations before the infusion. EDTA-blood samples were collected before and at 15, 30, 45, 60, 90, 120, 180, 240, and 360 min as well as 24 h and 48 h after infusion. The samples for 24 h and 48 h were collected after overnight fasting (8:00 a.m.). Samples were stored at -80 °C until further analysis. During the study day, subjects rested in a

supine position and were monitored continuously (electrocardiogram, heart rate, non-invasive blood pressure, and temperature). Subjects were allowed to drink water during the study day and eat after completion of the 6 h blood sampling.

3.7 ANALYSES

3.7.1 HPLC (Paper I-III)

An isocratic reversed-phase high-performance liquid chromatography (HPLC) including a dual-piston, high-pressure liquid delivery pump (LC-10AD VP; Shimadzu Corporation, Kyoto, Japan) and a C18 column (ReproSil-Pur, 4 × 100 mm; Dr. Maisch GmbH, Ammerbuch, Germany) were used to determine the concentration of kynurenine and KYNA. A mobile phase (50 mM sodium acetate, pH 6.2, 7 % acetonitrile) was pumped through the reversed-phase column at a flow rate of 0.5 ml/min. For KYNA, a second mobile phase (0.5 M zinc acetate) was delivered by a pump (P-500; Pharmacia, Uppsala, Sweden) post-columnar at a flow rate of 10 ml/h. Samples (50 µl) were manually injected to the loop (100 µl) of the injector (Rheodyne® 7725i; IDEX, Oak Harbor, WA, USA). Kynurenine (360 nm) was detected with a spectrometer (SPD-10A UV-VIS, Shimadzu Corporation) and KYNA (excitation 344 nm and emission 398 nm) was analyzed with a fluorescence detector (18 nm bandwidth, Jasco Ltd., Hachioji City, Japan). Detectors' signals were analyzed using the Datalys Azur software (Grenoble, France). Retention times: kynurenine: 4 min, KYNA: 7 min.

For determination of dopamine, DOPAC, HVA, 5-HIAA, and 5-HT, the mobile phase (70 mM sodium acetate, pH 4.1, 20 % methanol with 1.5 mM octanesulfonic acid and 0.01 mM Na_2EDTA) was delivered by a pump (LC-20AD; Shimadzu Corporation) through a column (4.6 9 150 mm, ZORBAX Eclipse XDB-C18; Agilent Technologies, CA, USA) at a rate of 0.68 ml/min. Samples (50 µl) were quantified in a high-sensitivity analytical cell (ESA 5011; ESA Inc., Chelmsford, MA, USA) electrochemically (sequential oxidation and reduction) controlled by a potentiostat (500 mV, Coulochem III; ESA Inc.). Signals from the detector were analysed using Clarity software (DataApex; Prague, Czech Republic). Retention times: dopamine: 6 min, DOPAC: 4 min, HVA: 7 min, 5-HT: 11.5 min, 5-HIAA: 5.5 min.

3.7.2 UPLC-MS/MS (Paper IV, V)

The UPLC-MS/MS system (Xevo TQ-XS triple quadrupole mass spectrometer; Waters, Manchester, UK) equipped with a Z-spray electrospray interface and a FTN system (Waters Acquity UPLC I-Class; Waters, Milford, MA), was operated in electrospray positive multiple reaction monitoring (MRM) mode. Conditions for the interface: detector gain 1, capillary 3.0 kV, source 150 °C, desolvation 650 °C, gas flow rate; desolvation: 1000 l/h, cone: 150 l/h. Conditions for the UPLC system: column 50 °C (Acquity HSS T3; 1.8 µm, 2.1 × 150 mm; Waters, part #: 186003540), guard column (Vanguard HSS T3; 1.8 µm, 2.1 × 50 mm column; Waters, part #: 186003976), mobile phase A (0.6 % formic acid in water), mobile phase B (0.6

% formic acid in methanol). Flow rate: 0.3 ml/min, run time for each sample: 13 min. Autosampler: 4 °C.

The software Masslynx 4.1 was used for calculating the dwell times for the MRM channels (data points 15-20) and the m/z for the MRM transitions (each individual analyte), along with optimal cone voltages and collision energies (determined by manual tuning using the instrument's built-in fluidics system). Peak intensities in MRM transitions (m/z), recorded at previously determined retention times and optimized instrumental settings were: Tryptophan (206→118 and 146, 7.01 min), Tryptophan-d_3 (208.1→118.8, 7 min), kynurenine (209→94 and 146, 5.76 min), kynurenine-d_4 (213→94, 5.7 min), KYNA (190.1→116 and 144, 7.99 min), KYNA-d_5 (195→121, 7.89 min), QUIN (168.1→78 and 124, 2.94 min), QUIN-d_3 (171→81, 2.86 min), PIC (123.9→78 and 96, 2.17 min), PIC-d_4 (128→82, 2.14 min).

3.7.3 mRNA expression analysis (Paper III)

Gene expression analysis was performed as previously described (Agudelo et al., 2014). In brief, Isol-RNA Lysis Reagent was used for extracting total RNA from brain hemispheres, according to manufacturer's instructions. Subsequently, RNA was used for cDNA preparation with the Applied Biosystem Reverse Transcription Kit after being treated with Amplification Grade DNase I. To quantify the mRNA levels cDNA was mixed with SYBR Green and PCR Master Mix. Quantitative Real-Time PCR was performed in a ViiA 7 thermal cycler. Analysis of gene expression was carried out with the ΔΔCt method and relative gene expression was normalized to hypoxanthine phosphoribosyltransferase (HPRT) or TATA sequence binding protein (TBP) mRNA levels. Gene expression analyses were presented as mRNA relative to controls. (For primer sequences used see paper III).

3.8 METHODOLOGICAL CONSIDERATIONS

3.8.1 Behavioural tests

Testing psychotic behaviour and especially positive symptoms of schizophrenia (hallucinations, delusions, disordered thoughts and speech) in animals is challenging. Few tests are available for rodents modelling behaviours relevant to positive symptoms. Existing tests are hyperactivity in response to novelty or stress (Geyer and Moghaddam, 2002; Young *et al.*, 2016), hyperlocomotion induced by psychostimulants (Staton and Solomon, 1984; Ikemoto, 2002) and prepulse inhibition (PPI) (Braff and Geyer, 1990; Bakshi and Geyer, 1998; Swerdlow *et al.*, 2001). In the studies presented herein, we have used the D-amphetamine-induced hyperlocomotion paradigm. Apart from the positive symptoms, also cognitive impairments belong to the symptomatology of schizophrenia. Working memory is the ability to rapidly store and manipulate new information and distinguish it from the old. Testing working memory is a way to evaluate cognitive dysfunctions. Many of the behavioural models relevant to schizophrenia focus on testing memory and learning.

Behavioural Test	Features	Symptom dimension	Result	Primary brain areas
Accelerated rotator (A, B)	Locomotion/ Baseline	n/a	=	Motor cortex, Co-ordination: cerebellum (1)
Open-field*	General locomotion	n/a	-	Motor cortex, Co-ordination: cerebellum (1)
Corner time	Anxiety/blunted affect	Negative	+	Hippocampus, Amygdala, Prefrontal cortex (2)
Center time	Anxiety/blunted affect	Negative	=	Hippocampus, Amygdala, Prefrontal cortex (2)
Y-maze (continuous alternations)*	Working memory	Cognitive	=	Hippocampus, Septum, Prefrontal cortex, Anterior cingulate cortex (3)
Radial 8-arm maze (B)	Memory	Cognitive		
	Working memory	Cognitive	=	Hippocampus, Septum, Prefrontal cortex, Premotor cortex, Anterior cingulate cortex (3)
	Reference memory	Cognitive	-	Posterior parietal cortex, Hippocampus, Entorhinal cortex (4)
D-amphetamine-induced hyperlocomotion*	Hyperactivity	Positive	+	Striatum, Nucleus accumbens (5)
Trace Fear Conditioning *	Cognition (fear learning)	Cognitive	Context/ Cue -/-	Hippocampus, Amygdala (6)
Pavlovian conditioning (A, B)	Cognition (associative learning)	Cognitive	-	Medial temporal lobe, Hippocampus, Prefrontal cortex, Striatum, Frontal motor related areas (7)
PPI (A, B)	Sensorimotor gating	Positive	=	CSPP circuitry ** (8)
Startle reactivity		Positive	-	

Table 1. Behavioural test reported in the literature using the dual LPS model. References are for primary brain areas involved in the testing. *tests included in this thesis, **CSPP circuitry: limbic cortex, striatum, pallidum and pontine tegmentum

A: (Oliveros *et al.*, 2017), B: (Peyton *et al.*, 2019), 1: (Heindorf *et al.*, 2018; Darmohray *et al.*, 2019), 2: (McEwen *et al.*, 2016), 3: (Richman *et al.*, 1986; Zahrt *et al.*, 1997; Hughes, 2004; Bach *et al.*, 2008), 4: (Spieker *et al.*, 2012; Kim *et al.*, 2018), 5: (Staton and Solomon, 1984; Ikemoto, 2002), 6:(Maren, 2008), 7: (Brasted and Wise, 2004; Bach *et al.*, 2008), 8: (Bakshi and Geyer, 1998; Swerdlow *et al.*, 2001).

Tests, such as trace fear conditioning and Y-maze (used for producing the results included in this thesis), are characterised by high face validity (Geyer and Moghaddam, 2002; Jones *et al.*, 2011). To model the negative features of schizophrenia, the third and last cluster of the disease's symptoms, researchers use paradigms that evaluate anxiety features. The test used here, evaluated time spent in the center of an open-field. In Table 1, all behavioural tests reported in the literature using the dual LPS model are summarized.

Together, the behavioural results suggest an effect on cognitive processes, more specifically long-term (reference) memory and/or memory recall and on psychosis-like behaviours related to increased striatal dopamine. However, given the literature findings regarding alterations in kynurenine pathway metabolites and enzyme expression in the prefrontal cortex, the testing battery could be expanded to include more specific tests of this brain region including tests of executive function and attention.

Locomotor activity test

The locomotor activity test is broadly used in animal behaviour studies to set the baseline level of motor activity, but also for detecting changes in locomotion used for validating models of schizophrenia and effects of antipsychotic treatments (Geyer and Moghaddam, 2002). Drug-induced hyperlocomotion is characterized by construct and face validity. Construct validity is defined by common features in neuropharmacological mechanisms among humans and rodents with different behavioural outcome. On the contrary, face validity is based on the fact that psychomotor agitation - a symptom in schizophrenia - is common between humans and rodents (Powell *et al.*, 2009). The concept of using locomotor hyperactivity for testing psychotic-like behaviour is based on the principle that induced dopaminergic activity in rodents results in motoric hyperactivity, which includes increased forward and/or horizontal locomotion, vertical activity or rearing and - in higher doses - stereotypy (Van Den Buuse, 2010). Additionally, clinical data support the concept of amphetamine-induced locomotion, showing that patients with schizophrenia are more sensitive to the effects of amphetamine, such as exaggerated stimulation of dopaminergic transmission (Laruelle *et al.*, 1996; Laruelle and Abi-Dargham, 1999; Perry *et al.*, 2009). The locomotor activity and specifically hypersensitivity to amphetamine has been used in our group to validate the effects of increased KYNA levels in rodent brain (Olsson *et al.*, 2012b; Liu *et al.*, 2014).

Trace fear conditioning

Fear conditioning is a behaviour highly conserved in many animal species including rodents as well as humans. Rodents have a species-specific response to fear that is called freezing and involves complete immobility excluding breathing. Fear conditioning is used for assessing memory and learning (Kim and Fanselow, 1992). It consists of a single learning session (conditioning), which causes behavioural changes that are robust and long lasting. The term fear conditioning covers two separate assays, cued and contextual fear conditioning

that are focusing on different neural processes. Cued fear conditioning is a type of Pavlovian conditioning that consists of an associative learning task in which mice learn to associate a neutral cue-tone stimulus with an aversive stimulus, a mild electrical foot shock. With context fear conditioning, we are assessing memory formation and recall, since animals learn to associate the shock with the training context such as the cage characteristics. The brain region that is involved in the acquisition and expression of conditional fear is the amygdala, while the hippocampus is involved in contextual fear conditioning. Both single and dual LPS administrations are connected with learning and memory deficits. A single LPS dose is linked to disruptions in contextual fear conditioning (Terrando *et al.*, 2010; Imbeault *et al.*, 2020) and repeated LPS administration to impaired Pavlovian conditioning (Oliveros *et al.*, 2017) and reference memory (Peyton *et al.*, 2019). Trace fear conditioning in our studies was implemented in the same way as in previous studies from our and other groups (Terrando *et al.*, 2010; Liu *et al.*, 2014; Imbeault *et al.*, 2020).

Y-maze

The Y-maze test is based on spontaneous alternation behaviour, a process assessing spatial learning and working memory, and is built on the explorative and curious nature of rodents (Deacon and Rawlins, 2006). The parts of the brain that are involved in the testing are mainly the hippocampus, the prefrontal cortex and the striatal circuits. The test is performed in a Y-shaped maze consisting of three identical arms having a 120° angle between them. Animals tested have free access to all three arms after entering the center of the maze. Rodents typically prefer to explore a new arm of the maze rather than re-entering the one already visited. Remembering which arm was just entered utilizes working memory. Mice with learning and memory deficits show lower spontaneous alternation behaviour (entries between arms) as a result of reduced ability to distinguish among previously seen and novel objects (Dudchenko, 2004).

3.8.2 Human primary fibroblasts

Fibroblasts are primary cells that can easily be obtained from individuals using a minimally invasive procedure. They are stable and easy to culture. Additionally, fibroblast-derived cell lines maintain their genetic stability for a long period, up to 15-20 passages (Hänzelmann *et al.*, 2015), they exhibit similarities at the molecular level with cells coming from the CNS (Kálmán *et al.*, 2016) and possess, to a high extent, signalling pathways as well as receptors and transcription factors similar to cells of neuronal origin (Rieske *et al.*, 2005). There are several reasons why we chose human primary dermal fibroblast cultures for establishing a cell model to complement our animal studies. Previous studies have shown that disease-related alterations of the kynurenine pathway in patients with schizophrenia may be reflected in patient-derived skin fibroblasts (Asp *et al.*, 2011; Johansson *et al.*, 2013). In our studies, we provide further support for the use of patient-derived fibroblasts as an essential tool to investigate the interplay between the immune system and the kynurenine pathway by

elucidating the mechanisms involved in this communication. This is clinically important as increased knowledge on this issue may enable the development of diagnostic and prognostic tools. Since fibroblasts are relatively easy to obtain, they can be isolated from different groups of patients with various disorders, thereby potentially providing better medication for these patients, involving a personalized treatment approach. Skin biopsy-derived fibroblasts originating from a well-characterized cohort of patients, such as our KaSP project, allow cell biological and functional comparisons between patients and healthy controls, and the possibility to store patient-derived and control cells in a bio-bank for future analysis. Such a bio-bank would allow evaluating possible biomarkers and pharmacological targets for psychiatric disorders.

Obviously, the relatively simplistic fibroblast model comes with a series of limitations. Most importantly, fibroblasts do not primarily represent the cell types crucially involved in psychiatric disorders, such as CNS neurons or glia. This obstacle can, at least in part, be circumvented by the establishment of induced pluripotent stem cells (iPSCs) from skin biopsies. Furthermore, subsequent differentiation into relevant cell types such as neurons and astrocytes, as well as the establishment of 3D cultures or organoids could represent sophisticated *in vitro* models for studying disease-relevant molecular mechanisms. In this way, potential traits of the individual patient relevant to the disease may be correlated to specific biological aberrations in the particular cell types studied. However, we need to keep in mind that the use of dermal fibroblasts alone hardly reflects a realistic situation when investigating the pathophysiology of multifactorial diseases such as psychiatric disorders. Such a simplistic cell model is rather used to investigate mechanistic aspects of a complex system and increase the understanding of the etiology of these disorders.

3.8.3 Human LPS model

In order to bridge our experimental findings (animal and cell culture) with human research, we used a well-established human *in vivo* model of systemic inflammation. This translational model of experimental human endotoxemia induced by intravenous administration of LPS elicits a transient, controlled, reproducible, and well-tolerated systemic inflammatory response. The dose used in the literature for the LPS challenge in humans ranges between 0.4 ng/kg to 4 ng/kg. Such doses are safe and without any known long-term health risks for the participating subjects. During the first 2-4 h, LPS infusion is clinically characterized by increased temperature, chills, fatigue, myalgia, backache, headache, and nausea (flu-like symptoms), as well as decreased blood pressure, tachycardia, and tachypnea. In addition, LPS affects mood and alters cognition in human subjects (Benson *et al.*, 2017; Engler *et al.*, 2017; Kotulla *et al.*, 2018).

3.8.4 Chromatography methods

HPLC is a method where the compounds are determined based on their retention time through a column and detected by a fluorescent, UV or electrochemical detector that measures the intensity of absorbance. The ultra high-performance liquid chromatography-Mass spectrometry (UPLC-MS/MS) method is a combination of HPLC with MS, where the different compounds are ionized and separated according to their mass/charge ratio by a mass analyser. The combination of the two analytical methods improves accuracy and sensitivity and also reduces experimental error.

Traditionally, our group has used HPLC for the analysis of tryptophan, KYNA and kynurenine. The UPLC-MS/MS was introduced in the laboratory recently and provides the opportunity to analyse several kynurenine pathway metabolites simultaneously. A side-by-side comparison using identical samples for HPLC and UPLC-MS/MS, clearly show that the methods yield highly comparable data for KYNA (Figure 4).

The selectivity, specificity, sensitivity, linearity, precision, accuracy, and matrix effect of the LC-MS/MS method used for measuring tryptophan and kynurenine metabolites has been validated by following the guidelines for bioanalytical method validation from the US FDA (https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf) and the EMA (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf) (Schwieler *et al.*, 2020) (Trepci et al., 2020 in press). In brief, all metabolites measured in the present thesis (tryptophan, kynurenine, KYNA, QUIN) are stable for up to four freeze-thaw cycles with less than 5 % variation and up to 4 h on bench at room temperature with less than 10 % variation. The lowest level of detection (S/N ratio of three) and the lowest level of quantification (S/N ratio of ten) are: tryptophan (10/10 μM), kynurenine (0.1/0.25 nM), KYNA (0.1/0.5 nM), QUIN (2.5/5.0 nM).

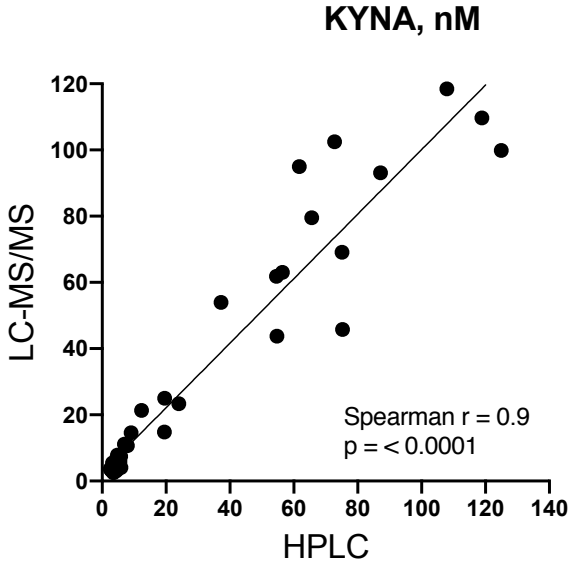

Figure 4. Correlation of KYNA levels analyzed by LC/MS and HPLC. Samples are from the human primary dermal fibroblast (**paper IV**).

3.9 DATA ANALYSIS - STATISTICS

For **papers I-IV,** statistical analyses were done in Prism (GraphPad Software Inc.), and data are presented, as mean ± SEM. P values below 0.05 are considered statistically significant. Data were analyzed using one-way (paper I), or two-way ANOVA followed by *post-hoc* Bonferroni's (**papers I-III**) or Sidak's (**paper IV**) multiple comparisons or unpaired *t*-test (**paper II, III**). Grubbs' test was used for identify outliers (**paper II**).

Statistical analyses for **paper V** were carried out using R programming language (R version 4.0.2). The alpha-level of significance was set at 0.05. P values below 0.005 were considered statistically significant. For each time point, a linear mixed-effect model was performed. Data were analyzed using post-hoc Bonferroni's correction. A linear mixed-effect model was also used to assess correlations.

4 RESULTS

4.1 MODELS OF INFLAMMATION

The overall scope of the studies included in this thesis was to investigate the interplay between inflammation and the kynurenine pathway. For that purpose, LPS was used in doses that induce a low-grade inflammatory response for both animals and humans, adjusted to the needs of each study. For our cell model, we used a cytokine model known to induce the kynurenine pathway.

4.1.1 LPS administration in rodents (Paper I-III)

Experimental inflammation in response to administration of LPS results in behavioural changes lasting long after the sickness behaviour has passed. As described above, single administration of LPS induces depressive-like behaviour through activation of the kynurenine pathway and increased QUIN (O'Connor *et al.*, 2009; Salazar *et al.*, 2012; Walker *et al.*, 2013; Tao *et al.*, 2020). Our findings here show that dual administration of LPS provides a more robust induction of the kynurenine pathway and that the kynurenine pathway metabolite profile is altered in a different manner than by a single administration of LPS.

4.1.1.1 Dual administration of LPS differentially alters brain and peripheral levels of kynurenine pathway metabolites (Paper I)

The increase in KYNA observed in psychiatric disorders is associated with an increase in proinflammatory cytokines (Lindqvist *et al.*, 2009; Söderlund *et al.*, 2009, 2011; Schwieler *et al.*, 2015). In order to establish an animal model recapitulating this scenario we aimed to elicit a systemic, low-grade inflammation in rodents. During the optimization phase of the LPS treatment protocol in mice, we realized that a single injection of LPS effectively induced the kynurenine pathway in the brain and led to increased production of the neurotoxic metabolite QUIN, but hardly any changes were observed in the levels of the neuroprotectant metabolite KYNA (Figure 5).

In **paper I**, we used dual injections of LPS (0.83 mg/kg + 0.83 mg/kg, i.p.), administered 16 h apart. Dual injections of LPS induced the kynurenine pathway distinctly and resulted in increased cerebral levels of kynurenine and QUIN as well as KYNA (Figure 5A, C, E). In the periphery, we found increased levels of kynurenine, decreased levels of KYNA and unaffected levels of QUIN (Figure 5B, D, F). To control that the observed changes following dual LPS injections were not the result of the double amount of LPS applied, we further analysed kynurenine levels after injection of 1.66 mg/kg LPS. Interestingly, we found that the increase in brain KYNA was a consequence of repeated exposure to LPS, rather than administration of a higher dose (Figure 6). The protocol emerging from this study emphasizes that two injections of LPS induce the kynurenine pathway more efficiently than a single exposure. This finding is in agreement with a previous study, showing that repeated injections of LPS are required to elicit a pronounced inflammatory response (Erickson and Banks, 2011). Interestingly, the

results following dual injection of LPS reflect the situation in subjects with schizophrenia, where increased KYNA levels are detected in the brain (Erhardt *et al.*, 2001; Schwarcz *et al.*, 2001; Nilsson *et al.*, 2005; Sathyasaikumar *et al.*, 2011; Linderholm *et al.*, 2012) and decreased levels are found in the periphery when compared to healthy individuals (Myint *et al.*, 2011).

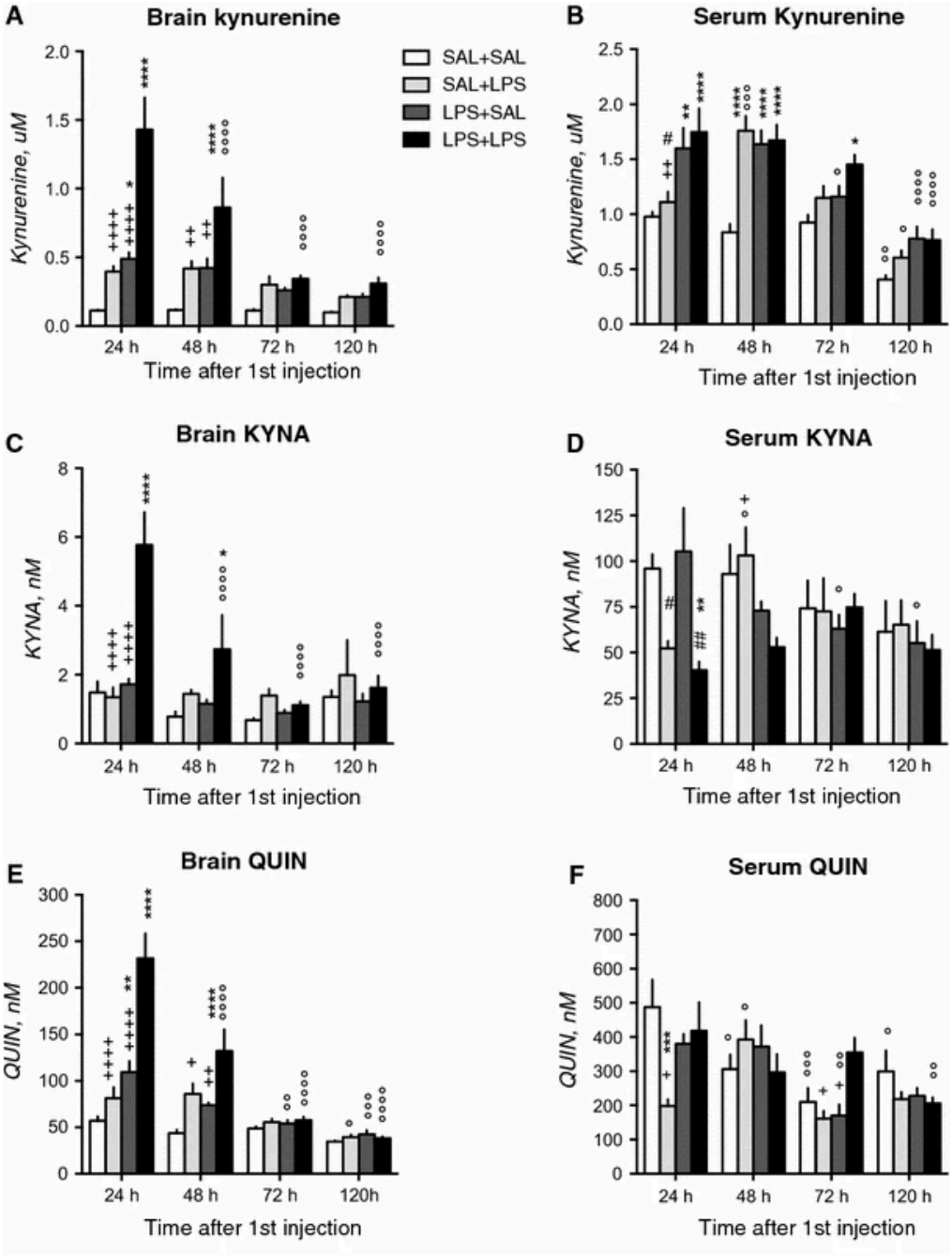

Figure 5 Kynurenine (A, B), Kynurenic acid (KYNA, C, D) and Quinolinic acid (QUIN, E, F) levels in mouse brain (A, C, E) and serum (B, D, F) after single (0.83 mg/kg) or dual (2 × 0.83 mg/kg) LPS injection. Mice were sacrificed up to 120 h after the first injection. n = 5-8 in each group. Levels are mean ± SEM. Two-way ANOVA, *post hoc* Bonferroni. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p ≤ 0.05 versus SAL + SAL; ++++ p < 0.0001, +++ p < 0.001, ++ p < 0.01, + p ≤ 0.05 versus LPS + LPS; ## p < 0.01, # p ≤ 0.05 versus LPS + SAL; °°°° p < 0.0001, °°° p < 0.001, °° p < 0.01, ° p ≤ 0.05 versus 24 h.

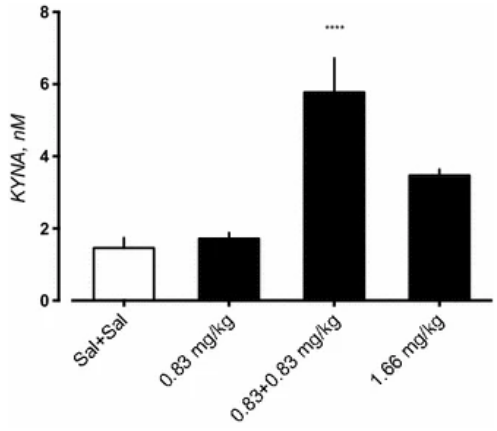

Figure 6. KYNA levels in mouse brain after single (0.83 mg/kg or 1.66 mg/kg) or dual (2 × 0.83 mg/kg) LPS injection. Mice were sacrificed 24 h after the first injection. n = 3-8 in each group. Levels are mean ± SEM. One-way ANOVA, *post hoc* Bonferroni. ****p < 0.0001.

In **paper I**, we also analysed monoamines and their metabolites in the brain. Dopamine and its metabolites HVA and DOPAC as well as serotonin (5-HT) and its metabolite 5-HIIA were analysed. As reflected by increased HVA:Dopamine and 5-HIAA:5-HT ratios after dual LPS injections (24 h and 48 h) and 24 h after a single LPS injection, we suggest that the turnover of dopamine and serotonin is affected by LPS (Figure 7).

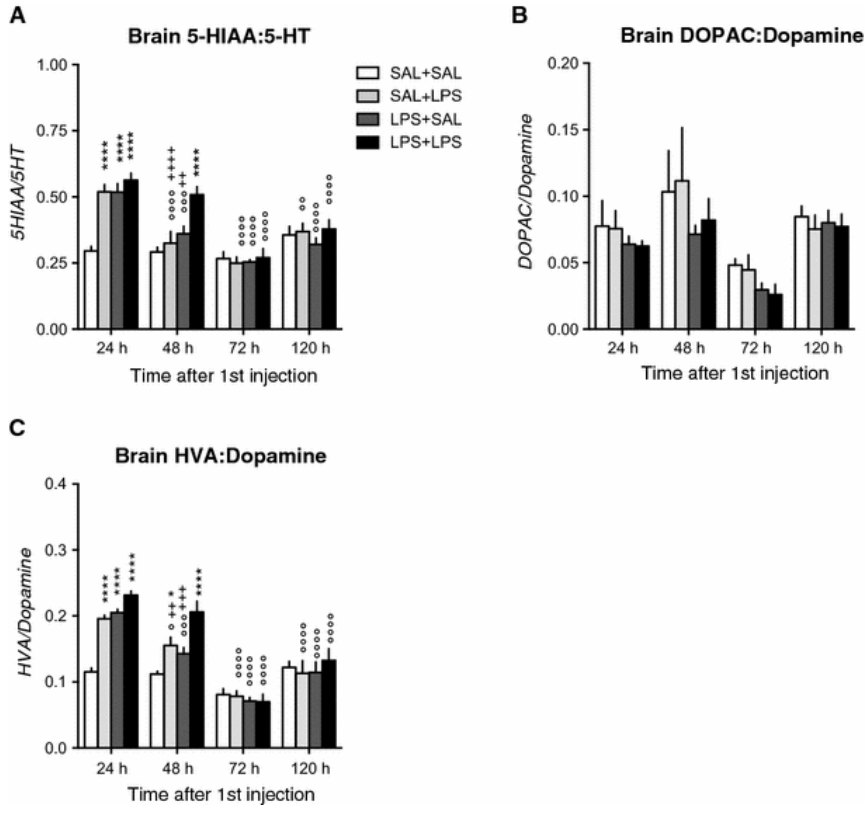

Figure 7. Metabolite concenration ratios in the brain: 5-HT:5-HIAA (A), DOPAC:Dopamine (B), and HVA:Dopamine (C) after a single (0.83 mg/kg) or dual (2 × 0.83 mg/kg) LPS injection. Mice were sacrificed up to 120 h after the first injection. n = 5-8 per group. Levels are mean ± SEM. Two-way ANOVA, *post hoc* Bonferroni. ****p < 0.0001, *p ≤ 0.05 versus SAL + SAL; ++++ p < 0.0001, +++ p < 0.001, ++ p < 0.01, versus LPS + LPS; ## p < 0.01, # p ≤ 0.05 versus LPS + SAL; °°°° p < 0.0001, °°° p < 0.001, °° p < 0.01, °p ≤ 0.05 versus 24 h.

So far, only few studies have analysed monoamines concentrations in the brain following LPS treatment. One study measured dopamine and its metabolites using *in vivo* microdialysis showing that HVA increased after a single LPS injection (Van Heesch *et al.*, 2014).

4.1.1.2 Alterations in the kynurenine pathway in response to dual LPS administration lead to behavioural impairments (Paper II)

After establishing the protocol for dual LPS administration and clarifying how this protocol alters the pattern of kynurenine pathway metabolites in the brain and periphery, we were interested in investigating the impact of an activation of the immune system by LPS on the behaviour of mice with emphasis on the aspects of behaviour reflecting psychotic-like behaviour and cognitive deficits. In order to address this question, we used amphetamine-induced locomotor activity, trace fear conditioning and the Y-maze tests.

We found that mice that received two LPS injections (0.83 mg/kg + 0.83 mg/kg, i.p.) were more sensitive to amphetamine and showed increased locomotion, compared to saline-treated control mice (Figure 8). These data are in line with previous studies, where rodents with elevated cerebral KYNA levels are hypersensitive to amphetamine (Olsson *et al.*, 2012b; Liu *et al.*, 2014; Erhardt *et al.*, 2017a). Although amphetamine-induced hyperactivity is used as a model for psychiatric disorders, it was only recently that researchers used it in humans in a reverse-translational approach to show similarities between humans and rodents by quantification of specific locomotor and exploration profiles (Young *et al.*, 2016). Additionally our results are supported by positron emission tomography (PET) studies revealing that patients suffering from schizophrenia present an exaggerated response of the dopamine system after exposure to amphetamine (Laruelle *et al.*, 1996; Laruelle and Abi-Dargham, 1999; Perry *et al.*, 2009).

For fear conditioning, decreases in both contextual and cue-dependent freezing time were observed following dual LPS injections (0.83 mg/kg + 0.83 mg/kg, i.p.), which is in agreement with results from previous studies presenting learning and memory deficits induced by a single LPS injection (Pugh *et al.*, 1998; Imbeault *et al.*, 2020) as well as with studies showing that dual administration of LPS induces impairments in learning (Oliveros *et al.*, 2017; Peyton *et al.*, 2019). In the Y-maze test, we did not observe any significant changes for dual LPS treated mice in percent of spontaneous alternation, same arm returns or alternate arm returns, but a reduction in total arm entries (Figure 9). These results, in combination with a reduced locomotor activity observed in dual LPS treated mice during the habituation phase in the open field, indicate either an effect on general locomotion or are related to anxiety and/or reduced motivation for exploration. Although these latter behaviours were related to an LPS-induced depression model reported previously (Remus and Dantzer, 2016), they are also in line with negative symptoms of schizophrenia (Temmingh and Stein, 2015).

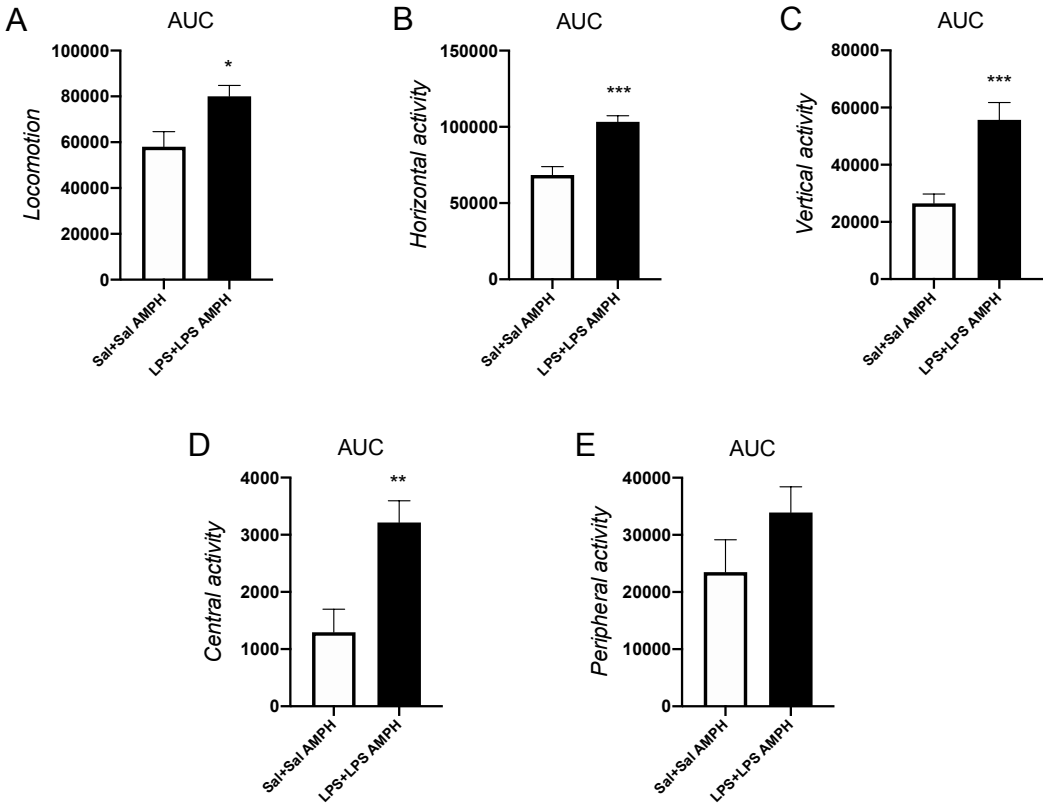

Figure 8 Dual-LPS-treated mice show increased motility in response to D-amphetamine. Area under the curve (AUC) was measured in response to D-amphetamine administration for A) Locomotion, B) Horizontal activity, C) vertical activity, D) central activity, and E) Peripheral activity. n=9-11 per group. *p<0.05, **p<0.01, ***p<0.001 unpaired t- test.

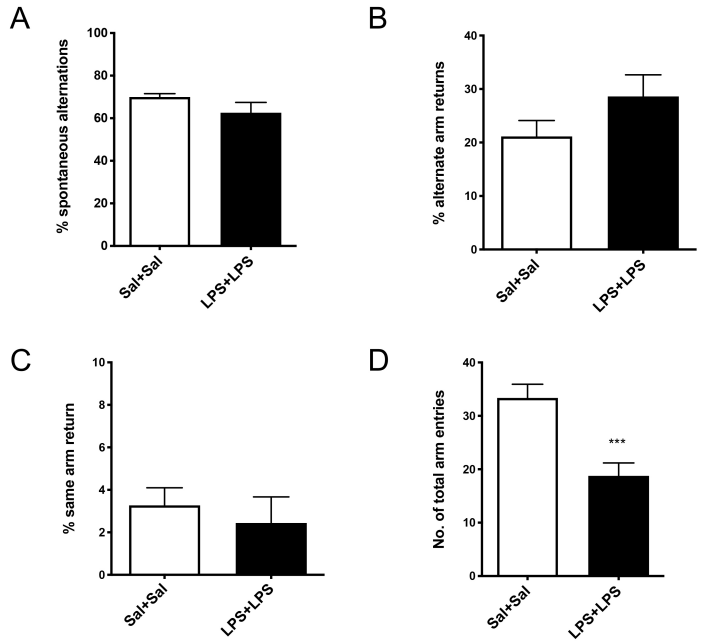

Figure 9. Performance in the Y-maze between dual-LPS-treated mice and controls. A) Percent spontaneous alternations, B) Percent alternate arm returns, C) Percent same arm returns, and D) number of total arm entries. n=9 per group. ***p<0.001 unpaired t-test.

4.1.1.3 Compensatory alterations in KAT expression in response to LPS (Paper III)

So far, our investigations clearly show that dual LPS administration increases KYNA levels in the mouse brain and induces behavioural impairments. This raised the question: are KAT II KO mice protected from the LPS-induced increase in brain KYNA levels? KAT II is the principal enzyme converting kynurenine to KYNA and its genetic ablation results in diminished ability to produce KYNA (Yu *et al.*, 2004; Potter *et al.*, 2010). Thus, one would expect that the loss of KAT II results in increased kynurenine and QUIN levels in the brain without an effect on cerebral KYNA levels following dual injection of LPS.

Consistent with our first study, WT mice showed increased brain levels of KYNA following dual injections of LPS (0.83 mg/kg + 0.83 mg/kg, i.p.), but unexpectedly, the same effect was observed in KAT II KO mice in both P 22 (Figure 10A) and adult mice (Figure 10B).

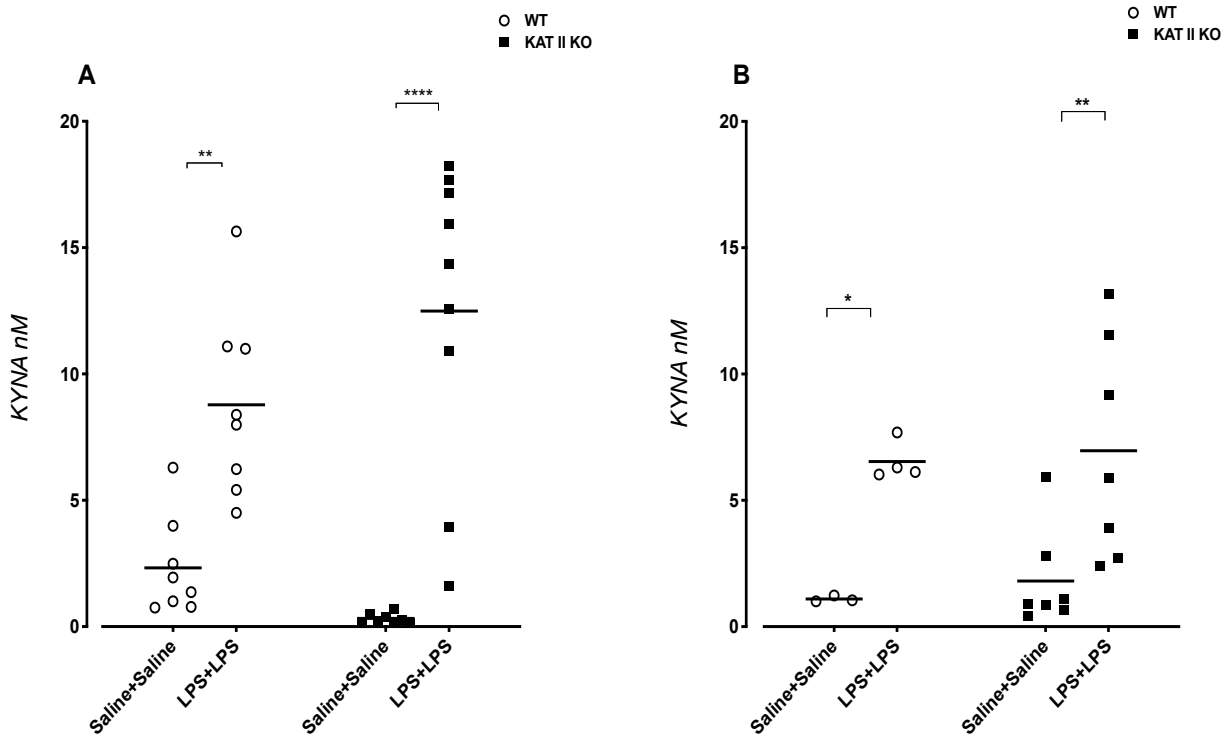

Figure 10. Brain KYNA levels after dual saline or LPS (2 × 0.83 mg/kg, i.p.) WT and KAT II KO mice A) at P 22 and B) in adulthood, n=3-9 per group. Data are presented as mean ± SEM and analysed with two-way ANOVA, *post hoc* Bonferroni. *p<0.05, **p<0.01, ****p<0.0001.

In order to investigate the mechanisms behind these results we measured mRNA expression levels of the four KATs. Interestingly, dual LPS injections resulted in the upregulation of KAT III mRNA levels, both in KAT II KO mice and their WT controls at P 22 (Figure 11) and in adult mice (Figure 12).

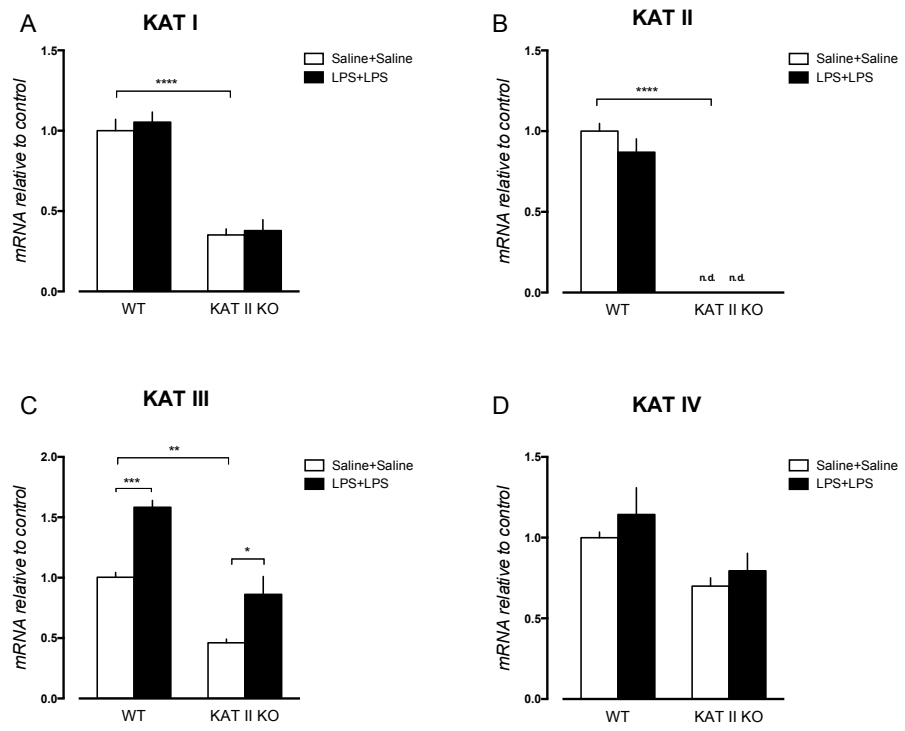

Figure 11. Brain mRNA expression levels of KAT I (A), KAT II (B), KAT III (C) and KAT IV (D) mRNA expression in the brain of in WT and KAT II KO mice at P 22 following dual saline or LPS injections (2 × 0.83 mg/kg, i.p.), n=6 per group. Data are presented as mean ± SEM and analysed using two-way ANOVA, *post hoc* Bonferroni. **p<0.01, ***p<0.001 ****p<0.0001.

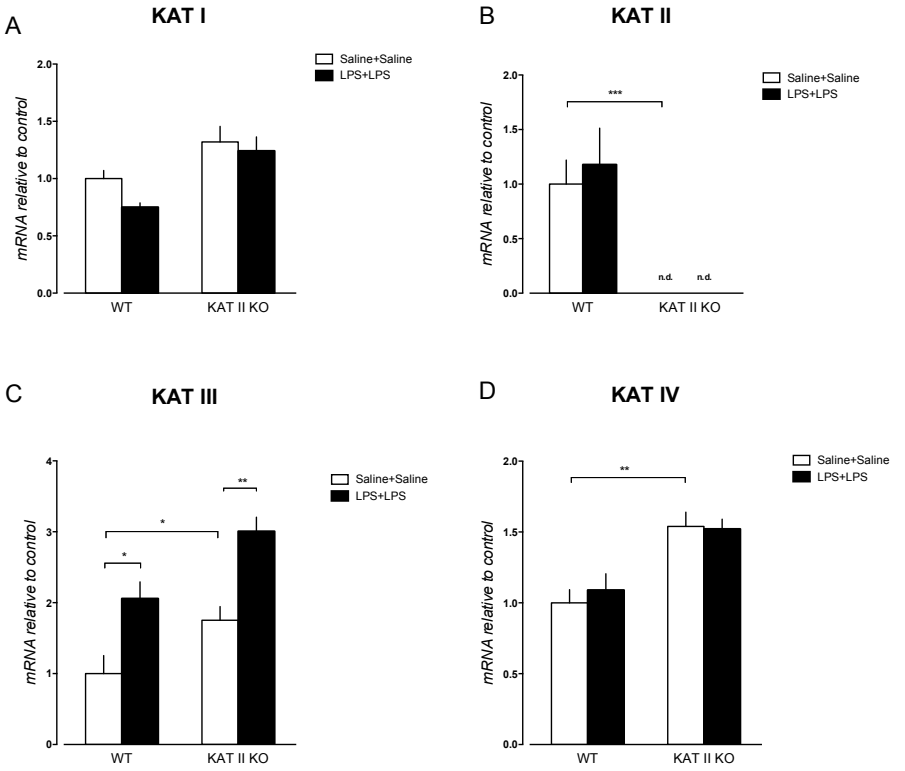

Figure 12. KAT I (A), KAT II (B), KAT III (C) and KAT IV (D) mRNA expression in the brain of adult WT and KAT II KO mice following dual saline or LPS injections (2 × 0.83 mg/kg, i.p), n=5-7. Data are presented as mean ± SEM and analyzed using two-way ANOVA, *post hoc* Bonferroni. *p<0.05, **p<0.01.

In order to exclude the possibility that the increase of kynurenine *per se* was responsible for the observed effects, we administered kynurenine (20 mg/kg or 40 mg/kg, i.p.). These doses of kynurenine increased KYNA production without inducing KAT III expression. Therefore, we conclude that the expression of KAT III is directly regulated by immune activation by LPS. In addition to the genetic ablation of KAT II, we investigated whether a selective KAT II inhibitor (PF-04859989) could inhibit KYNA formation induced by dual LPS injections. Our results clearly show that pharmacological inhibition of KAT II can reduce but cannot prevent brain KYNA production induced by dual LPS administration in WT or KAT II KO mice (at P 22 or adult age). Previous reports show that brain KYNA levels in KAT II KO mice stay low until P 28 but normalize in adulthood (Yu *et al.*, 2004), suggesting that the other KAT isoforms compensate for the absence of KAT II (Yu *et al.*, 2006). In particular, KAT III mRNA expression increased during development and reached the highest values in adulthood for both WT and KAT II KO mice (Yu *et al.*, 2006).

In **paper III** we report that dual LPS administration upregulates the expression of KAT III mRNA in both adulthood and at P 22 in WT mice and KAT II KO genotypes. Although all rodents share common features, we wanted to exclude the possibility that the induction of KAT III observed in mice after stimulation of the immune system by LPS originates from species-specific mechanism. Therefore, similar studies were performed in rats. In line with the results in mice, we found increased expression of KAT III mRNA and increased brain KYNA levels following dual injections of LPS (0.83 mg/kg + 0.83 mg/kg, i.p). Indeed, rat brain KYNA levels were maintained higher as a result of the dual LPS administration even after pre-treatment with the selective KAT II inhibitor (PF-04859989). Taken together, we pinpoint a specific role of KAT III in the neo-synthesis of KYNA during states of inflammation and suggest that KAT III is a potential target for antipsychotic and cognitive dysfunction interventions.

4.1.2 Cytokine Administration in Cells (Paper IV)

Our next aim was to explore how immune stimulation affects kynurenine pathway metabolites in an isolated cell system. Given the data obtained from the animal studies, we were also interested in exploring, whether a cytokine-induced increase in KYNA, is prevented by applying the selective KAT II inhibitor PF-04859989 (500 nM). To perform our studies, we chose to work with primary human fibroblasts. Among the different peripheral tissues, human fibroblasts are easy to obtain, to culture under controlled conditions, and to store. Fibroblast cultures have been used in previous studies as a model system to study psychiatric disorders (Manier *et al.*, 2000; Gassó *et al.*, 2014; Kálmán *et al.*, 2014; Mesdom *et al.*, 2019), which is an additional attraction of this cell model.

4.1.2.1 Cytokine stimulation in human primary fibroblasts (Paper IV)

Stimulation of human primary fibroblasts with IL-1β (10 ng/ml) did not affect extracellular levels of KYNA while IFN-γ (20 ng/ml) strongly increased the production of KYNA. Interestingly, the combination of IFN-γ (20 ng/ml)/IL-1β (10 ng/ml) exerted a synergistic effect resulting in an even higher extracellular concentration of KYNA (Figure 13). The IC_{50} value (*in vitro* enzymatic assay, (Kozak *et al.*, 2014)) for PF-04859989 is in the nanomolar range (23 nM) for KAT II inhibition and in the micromolar range for the other KATs (KAT I: 2.16 μM, KAT III: 10.7 μM and for KAT IV: > 50 μM. Administration of the KAT II inhibitor PF-04859989, in a dose high enough to effectively inhibit KAT II (500 nM), but still far below the IC_{50} values for the other KATs, decreased extracellular KYNA concentrations in both IFN-γ (20 ng/ml)- and IFN-γ (20 ng/ml)/IL-1β (10 ng/ml)-treated cells with less than 50 % decrease for the latter (Figure 13). The inability of PF-04859989 to completely block the cytokine-induced KYNA release further underlines the importance of other KAT enzymes in the production of KYNA during stimulation of the immune system.

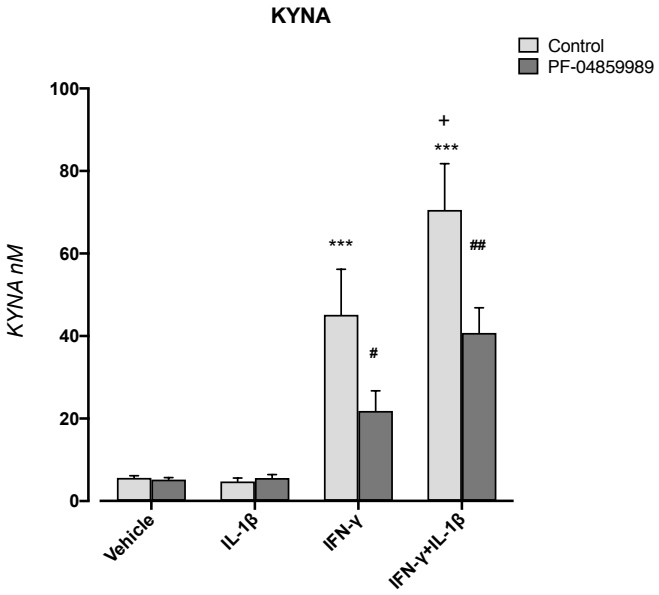

Figure 13. Extracellular KYNA levels in human, primary fibroblast cultures exposed to pro-inflammatory cytokines. Fibroblast cultures from healthy subjects (n=10) treated with vehicle, IL-1β (10 ng/ml), IFN-γ (20 ng/ml), or their combination for 48 h. PF-04859989 (500nM) was given 1 h prior to cytokine treatment. ***p<0.001 compared to the vehicle control group. $^{\#}$p<0.05, $^{\#\#}$p<0.01 versus control group, $^{+}$p<0.05, versus IFN-γ control group (two-way ANOVA, *post hoc* Sidak). Error bars provide standard error of the mean (SEM).

Of note, stimulation of primary human fibroblasts with IFN-γ (20 ng/ml) and IL-1β (10 ng/ml) or the combination of both, did not have an impact on the production of QUIN (Figure 14C). Further, pre-treatment with the KAT II inhibitor did not have any effect on QUIN levels. However, stimulation with IFN-γ (20 ng/ml) and IL-1β (10 ng/ml) or the combination of IFN-γ (20 ng/ml)/IL-1β (10 ng/ml) reduced tryptophan levels, indicating a larger consumption of tryptophan when more kynurenine and KYNA are produced (Figure 14A). Extracellular levels of kynurenine were not affected by the IL-1β treatment alone, but were increased by the IFN-γ stimulation as well as the combination of IFN-γ/IL-1β in a synergistic manner (Figure 14B).

Administration of the KAT II inhibitor did not affect the basal or the cytokine-induced kynurenine levels (Figure 14B), which is in line with the relative position of KAT II in the pathway downstream of kynurenine.

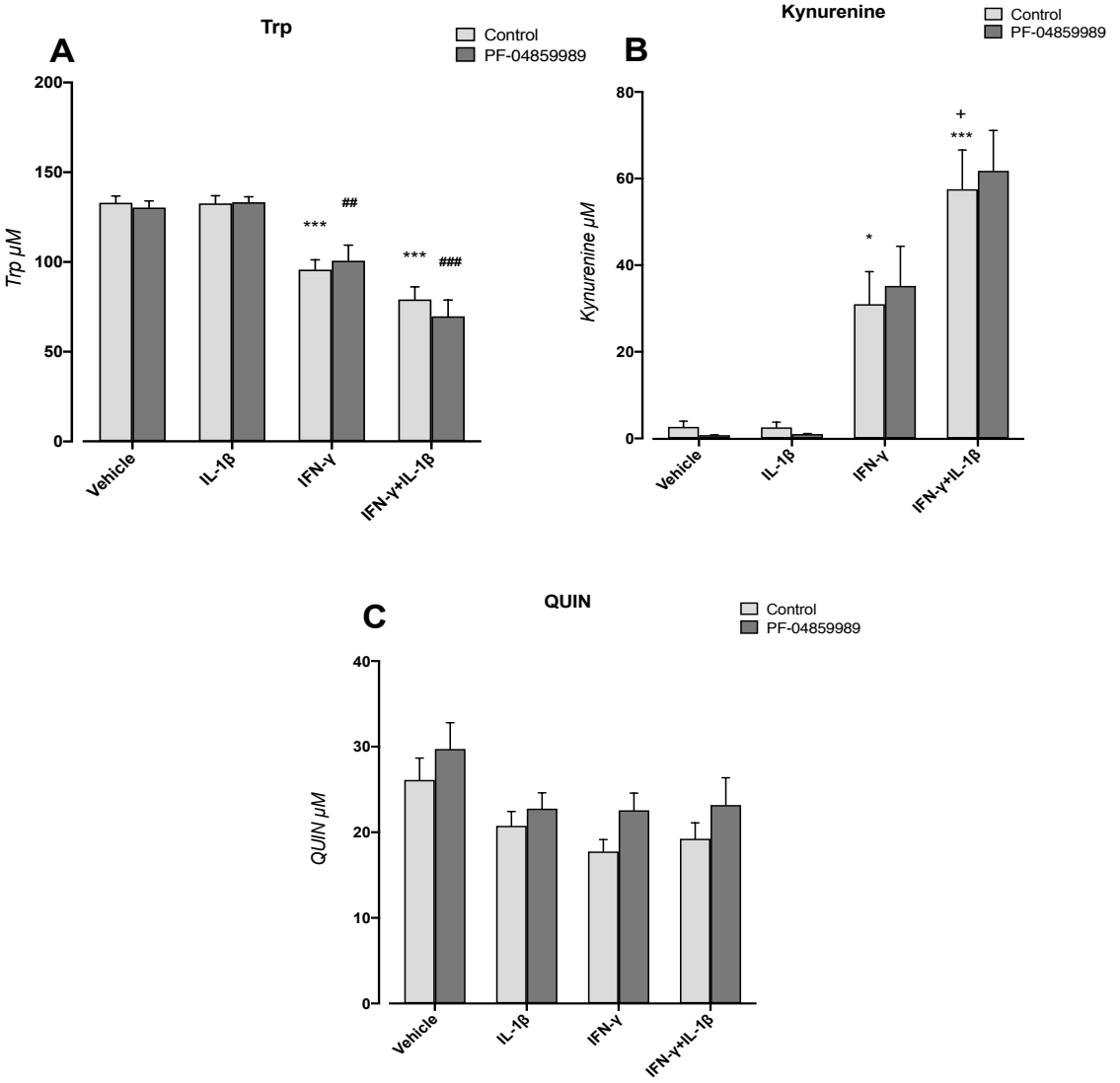

Figure 14. Extracellular levels of A) Tryptophan (Trp) B) kynurenine, C) QUIN in human, primary fibroblast cultures exposed to pro-inflammatory cytokines. Fibroblast cultures from healthy subjects (n=10) were treated with vehicle, IL-1β (10 ng/ml), IFN-γ (20 ng/ml), or their combination for 48 h. PF-04859989 (500nM) was given 1 h prior to cytokine treatment. *p<0.05, ***p<0.001 versus vehicle control group, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.01 versus PF-04859989 treated control group, $^{+}$p<0.05, versus IFN-γ control group (two-way ANOVA, *post hoc* Sidak). Error bars provide standard error of the mean (SEM).

In agreement with the main topic of this thesis, we also used LPS as a pro-inflammatory cue for the stimulation of human primary fibroblasts. Much to our surprise, neither single nor double stimulation at various doses (10, 20, 40, 200 ng/ml) of LPS elicited any change in extracellular KYNA levels. Obviously, fibroblasts are not *bona fide* immune cells and – even though they express TLR4 and react to LPS stimulation (Yao *et al.*, 2015; Bhattacharyya *et al.*, 2016; Li *et al.*, 2019) – different pro-inflammatory cues can elicit qualitatively different

responses in different cell types. In summary, inhibition of KAT II reduced the cytokine-induced production of KYNA by approximately 40 % in human fibroblasts, suggesting that the increase in KYNA production following cytokine exposure highly depends on KATs other than KAT II.

4.1.3 LPS Administration In Humans (Paper V)

The translational model of experimental human endotoxemia induced by intravenous administration of LPS elicits a transient, controlled, reproducible, and well-tolerated systemic inflammatory response. Systemic intravenous administration of LPS (2 ng/kg) evokes a transient inflammatory response (Heinzl *et al.*, 2020) and is associated with activation of both the neurotoxic and the neuroprotective branch of the kynurenine pathway. The present results show that administration of LPS (2 ng/kg) increased the ratios of tryptophan/ kynurenine, KYNA/ kynurenine and QUIN/ kynurenine (Figure 15) indicating activation of KMO, kynureninase and of KATs.

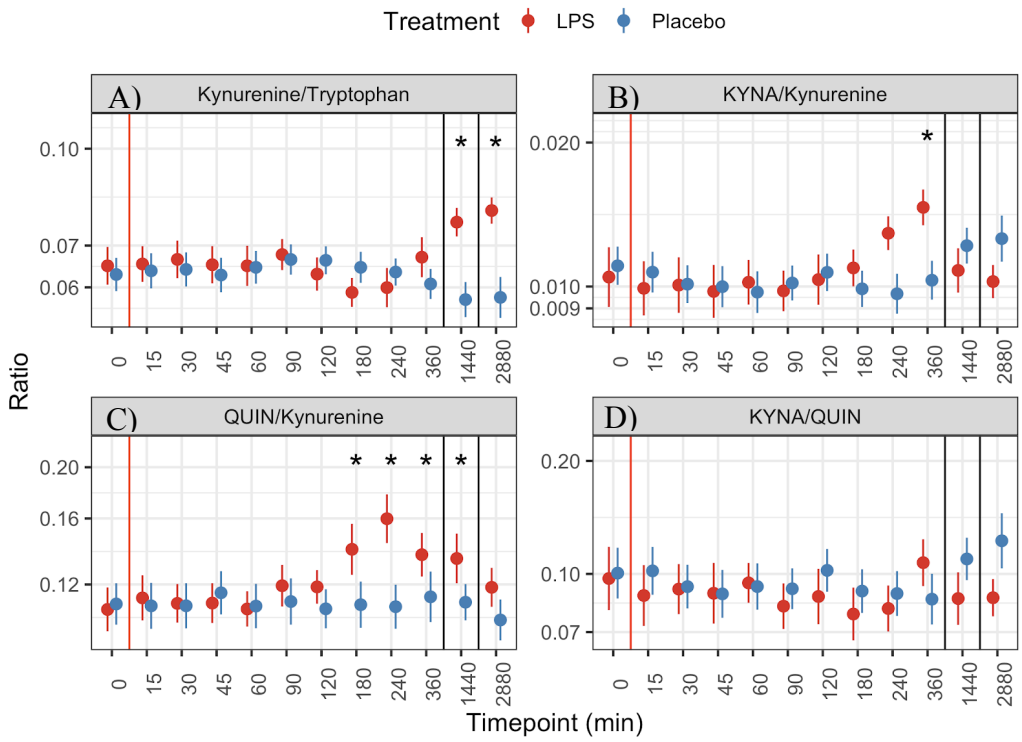

Figure 15. Ratios of metabolite concentrations after LPS (red) or placebo (blue) injection. The red line indicates the timepoint of LPS injection injection, the black lines the change between the three consecutive days. Data is presented as mean and standard error of the mean. *p<0.005.

Even though the results from this study are not sufficient to conclude direct causality, the associations between the metabolite ratios and levels of the inflammatory markers, IL-6 and

CRP, support the importance of the inflammatory response as an activator of the kynurenine pathway (Figure 16).

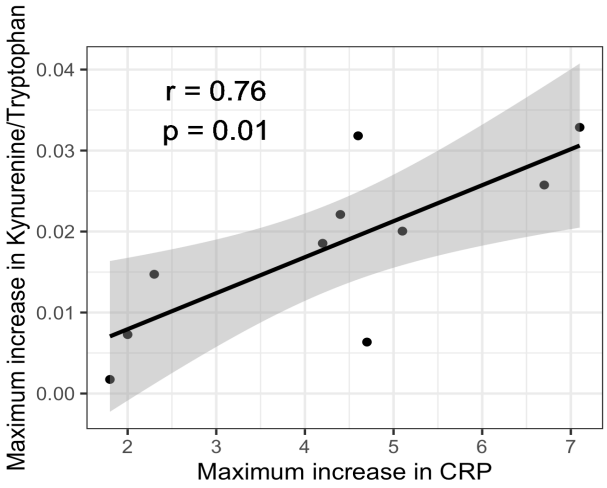

Figure 16. Correlation between the maximum increase in CRP and the maximum increase in the kynurenine/tryptophan ratio independent of time. The black line indicates the conditional mean, the grey area indicates the 95 % confidence interval.

The aim of the present study was to investigate whether acute, intravenous LPS administration, given in a dose (2 ng/kg body weight, *Escherichia coli* O113) evoking a substantial inflammatory response (Heinzl et al., 2019) affects the plasma concentration of kynurenine metabolites in healthy human subjects. This dose was found to increase the kynurenine/tryptophan ratio and showed that the pathway is active for at least 48 h post LPS-injection. The lack of increase in specific kynurenine pathway metabolite concentrations is in contrast to a recent study showing that LPS (0.8 ng/kg body weight, *Escherichia coli* group O:113) increases plasma kynurenine and KYNA 2 h and 6 h, respectively, post-injection (Kruse et al., 2019). The reason for this discrepancy is unclear but might be related to different batches of LPS. It might also be related to our small sample size and the fact that we only included young male healthy subjects. Thus, our study might not have had the power to detect smaller effect sizes, even if this limitation is partially counterbalanced by the cross-over design.

5 GENERAL DISCUSSION

As a source of several neuroactive metabolites, the kynurenine pathway serves as a link between immune signalling and brain neurotransmission (Söderlund *et al.*, 2011; Mándi and Vécsei, 2012). The present thesis, utilizing approaches ranging from biochemical analysis to human studies, serves to validate this link, i.e. that immune signalling can activate the kynurenine pathway and lead to the manifestation of aberrant behaviours also seen in psychiatric diseases. Most importantly, dual administration of LPS in rodents recapitulates many biochemical and behavioural features related to human psychotic disorders and cognitive dysfunctions and further emphasizes the importance of immune components in their pathology. We show that dual but not single administration of LPS increases KYNA levels in the brain while peripheral levels of the compound are decreased. Furthermore, our data show that dual LPS administration increases cerebral tryptophan, as well as the turnover of serotonin and dopamine. The finding that peripheral levels of KYNA are decreased while brain KYNA and dopamine turnover are increased is interesting with regard to psychotic disorders. Although the discrepancy between immune-induced effects on KYNA levels in the CSF versus peripheral tissues remains obscure, KYNA levels in serum seem to be lower (Myint *et al.*, 2011; Wurfel *et al.*, 2017), while brain levels of KYNA are higher in subjects with schizophrenia (Erhardt *et al.*, 2001; Schwarcz *et al.*, 2001; Nilsson *et al.*, 2005; Linderholm *et al.*, 2012) compared to healthy controls.

Interestingly, animal models of psychotic disorders show increased sensitivity to amphetamine (see Young et al., 2016). Indeed, enhanced sensitivity to amphetamine is also seen in animal models where cerebral KYNA is elevated (Olsson *et al.*, 2012b; Liu *et al.*, 2014; Erhardt *et al.*, 2017a). These findings are also supported by human data, where several studies have shown that subjects with schizophrenia are more sensitive to amphetamine than healthy subjects (Laruelle *et al.*, 1996; Laruelle and Abi-Dargham, 1999; Perry *et al.*, 2009). In line with these data, and in accordance with the increased turnover of dopamine found in **paper I**, we report in **paper II** that dual LPS-treated mice are more sensitive to amphetamine-induced hyperlocomotion. Although the findings of reduced spontaneous movements, as well as the reduced total arm entries in the Y-maze, could be a result of locomotor issues, they might also indicate anxiety and/or a reduced motivation to explore in dual LPS treated mice. Moreover, in line with our previous studies on behaviour following dual LPS treatment (Oliveros *et al.*, 2017; Peyton *et al.*, 2019), we also found cognitive deficits associated with learning in these mice. Therefore we conclude that dual LPS treatment in mice produces a robust induction of the kynurenine pathway, including an increase in KYNA levels in the brain. LPS-induced activation of the immune system causes cognitive deficits and enhances dopaminergic signalling, both of which are important hallmarks of schizophrenia and psychosis. Thus, the dual LPS model shows both face and construct validity as it adds biochemical and behavioural aspects indicative of psychotic disorders. In **paper III**, we aimed to investigate whether ablation of KAT II, either by using KAT II KO mice or by administration of a specific KAT II inhibitor, can protect from LPS-induced elevation of brain KYNA. Previous findings have shown that KAT II is the most important enzyme for KYNA

biosynthesis under physiological conditions in the mammalian brain (Schmidt *et al*., 1993; Guidetti *et al*., 1997). Unexpectedly, KAT II KO mice, as well as mice treated with a selective KAT II inhibitor, also displayed increased brain KYNA levels following dual LPS treatment. We then set out to investigate the mechanisms behind this increase in brain KYNA following dual LPS treatment. Here, mRNA expression levels of the four KAT enzymes were measured, and notably, only the expression of KAT III was upregulated by dual LPS injections. This effect was observed in both WT mice and KAT II KO mice and further confirmed in LPS-treated rats. These results pinpoint a specific role of KAT III in the neo-synthesis of KYNA during states of inflammation. Therefore, we hypothesise that KAT III is a potential target for interventions aimed at improving cognitive dysfunctions and reducing psychotic behaviours during states of inflammation, and we are currently developing drug-like molecules aiming at specifically inhibiting this enzyme. This drug discovery program is performed in collaboration with the Drug Discovery platform at Science for Life Laboratory (SciLifeLab). Indeed, one of the aims of the cell culture studies leading up to **paper IV** was to establish a stable cell culture model for investigating the efficacy of newly developed KAT III inhibitors for reducing immune response-induced synthesis of KYNA. In the present thesis, primary human fibroblast cultures from healthy subjects were used for studying how pro-inflammatory stimulation affects the kynurenine pathway. Interestingly, stimulation with pro-inflammatory cytokines increased the production of kynurenine and KYNA, whereas the synthesis of QUIN was not affected. Moreover, treating human fibroblasts with a selective KAT II inhibitor reduced the cytokine-induced production of KYNA with only approximately 40 %. Thus, these data confirm our findings in rodents suggesting that enzymes other than KAT II are of importance for the neo-synthesis of KYNA during inflammation. To translate the experimental results obtained in the present thesis to humans, we investigated, in **paper V,** the effects of systemic LPS administration on the kynurenine pathway in healthy human subjects. A single administration of LPS was found to activate both the neurotoxic and the neuroprotective branch of the kynurenine pathway for at least 48 h post LPS-injection. However, whereas plasma concentrations of kynurenine and QUIN were elevated in the LPS-treated group, no effect on KYNA levels was observed.

Overall, the results of the present thesis suggest that the dual LPS model can be used as an animal model for psychosis and cognitive deficits. The model shows face and construct validity regarding central and peripheral KYNA levels and reflects features of psychotic symptoms and cognitive impairment. In this regard, the model may be important for studying the involvement of inflammation in such conditions and can aid in the development of new treatment strategies. Thus, in combination with a cellular assay, the dual LPS model would be suitable for translational studies of novel immunomodulatory agents, aimed at diminishing KYNA synthesis in psychotic disorders.

Turning experimental findings into clinical reality may be challenging. There are numerous occasions where researchers did not manage to transfer protocols from preclinical studies to humans and there are also a lot of clinical trials that have failed to produce results expected from those accumulated in relevant preclinical experiments and non-primate animal models.

Therefore, selection of experimental human models is of utmost importance to translate hypotheses into man. In the present thesis, the human endotoxemia model did not exactly mirror the results from our experimental studies for each specific metabolite, but it indeed emphasized that challenging the immune system elicits essential changes in the kynurenine pathway. Repeated LPS administration in humans in doses resembling those for animal experiments, might be required to mirror the situation in schizophrenia, but may not be ethically defendable. Recently, new ways to further enhance immune activation by LPS have been tested in humans using continuous LPS administration (Kiers *et al.*, 2017). In this model, a bolus dose of LPS is followed by continuous LPS infusion over several hours. This treatment induces a more prolonged effect with higher cytokine concentrations, more circulating leukocytes, and an increased duration of fever and clinical symptoms. Possibly, the continuous LPS infusion model may mimic the condition of the inflammation seen in schizophrenia better and it would be interesting to analyse kynurenine pathway metabolites in this model. Before a human LPS model can be used for testing the efficacy and potency of newly developed inhibitors of different KATs, it will be essential to further study animal behaviour in relation to administration of LPS in order to strengthen the link between behavioural aberrations and the induction of the kynurenine pathway, KYNA levels and KAT III expression.

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